

Dingell encounters feather wall

Mr John Dingell's committee in the US House of Representatives has predictably made very little headway in its inquiry into the immunology of transgenic mice. But it is too soon to celebrate a famous victory for science.

REPRESENTATIVE John Dingell's subcommittee in the US House of Representatives has had a frustrating time in its search for scandal at the Whitehead Institute (see page 163). After two days of hearings, there is not much to say unless it is that formal congressional inquiries are not the best way of refereeing research reports. Dingell may have known that all along. His declared purpose in hearing public evidence about the antecedents of a single published research report was not so much to tell whether the conclusions drawn therein are correct, but to throw light on the scientific community's claim that it can be trusted to police its own affairs. But on its own recognizance, so to speak, the subcommittee misled itself by looking for quasi-judicial mechanisms for assessing people's quality and prudence, whereas the mechanisms that matter are informal. Good research reports may change the course of history, others are forgotten.

That is not to say that formal mechanisms for regulating quality and accuracy have no place in science. During the past few years, institutions as different as university departments and the National Institutes of Health (NIH) have been faced with the unhappy need to invent one formal device after another for dealing with allegations that published research is not what it seems. The Dingell committee is exercised that many of the committees established to look into particular incidents have been less than fully effective. That NIH are having to reopen the inquiry into the disputed paper dealt with at the Dingell hearings is unfortunate, to say the best of it. The moral for science is that when, for whatever reason, the informal mechanisms for telling what is good and what less good have to be replaced by more formal mechanisms for enquiring into allegations of more serious wrongdoing, the formal mechanisms should be rigorous and should be prosecuted with zeal.

For what it is worth, the disputed paper of which Dr David Baltimore is the most celebrated author should never have become the subject of an inquiry such as that of the past two weeks. Nobody has ever claimed that it was written and published with the intention to deceive. When Dr Margot O'Toole (who gave evidence last week) first complained that one of the reagents used in the study was not as specific as had been thought (and was afterwards claimed to be), she acted properly by raising the issue internally. It is not her fault that this claim afterwards became a *cause célèbre*. If Dingell's eventual report

should say that it is a black mark for science that O'Toole has been unjustly in the wilderness for the past three years, that will be only fair.

Baltimore's role in the affair, now often named after him, is similarly unnecessary. At the outset, it may have seemed natural that the most distinguished of the small group of authors of the disputed paper should have assumed responsibility for defending it against criticisms, first internal, then external. But that may have been mistaken: the laboratory bench is a great leveller, at which it is not always possible for a person to vouch that a colleague's work is as careful as his or her own. Baltimore seems also to have acted haughtily when Mr Walter Stewart and Dr Ned Feder, acting then as private citizens, embraced O'Toole's original complaints and multiplied them, for each, taken singly, is small.

The scientific community has rallied to Baltimore's defence; the Dingell committee will have been inundated with mail (not always flattering) this past week. The community's instincts are right: congressional committees do not make good referees, while, three years after the appearance of the disputed paper, it can hardly matter whether its conclusions are correct or otherwise. But the community, in defending one whom it rightly considers a true hero, should not make his mistake of defending what need not have been defended. The issue is not whether Baltimore and his colleagues were right in 1986, but whether they and others have the right in good faith to make mistakes. □

Deciding about bombs

There is just a week in which to find a Western compromise on nuclear weapons in Central Europe.

THE crunch is at hand on the issue of short-range missiles in Europe. Next week, there will be a meeting of the council of the North Atlantic Treaty Organization (NATO), attended by heads of government including President George Bush, at which West Germany's wish to open negotiations with the Warsaw Pact on short-range missiles will be under fire, chiefly from Britain and the United States. The following week, Mr Mikhail Gorbachev is due in Bonn, and can be relied upon to



sharpen the conflict between West Germany and its military allies. Mr Edvard Shevardnadze, the Soviet Foreign Minister, made that clear last week by saying that the Soviet Union would even consider scrapping the treaty on intermediate-range missiles (INF) signed at the end of 1987 unless there is also an agreement on short-range missiles.

It is especially disappointing that the intra-NATO row should have blown up when the prospects for further progress towards arms control in Europe should be brighter than ever. INF is a considerable prize, negotiations on conventional forces are under way at Vienna and Mr James Baker, the US Secretary of State, agreed last week in Moscow that the negotiations on strategic weapons, postponed for the duration of the US elections, should now be resumed. That promises to widen the area of agreement beyond Europe. That is why it must be hoped that the NATO's internecine squabble can be brought to a seemingly end next week.

The starting point for compromise should be a reassessment of the military balance in Central Europe. Since the rise of the doctrine of "flexible response" in the early 1960s, NATO has relied on the potential use of relatively short-range (100 km or less) nuclear weapons to counter the Warsaw Pact's superiority on the ground. Nuclear bombs fired from artillery pieces or short-range missiles would, on this view, be used to break up or destroy opposing forces massed in a conventional attack. The classic NATO view is that such uses need not lead inexorably to general all-out nuclear war; if the nuclear force is just sufficient for its immediate military purpose, nobody would have an interest in escalation. The two obvious caveats are that cool calculations of how much nuclear force is just enough are not best made by battlefield commanders and that West Germany would bear the brunt of the use of any nuclear weapons.

West Germany is also threatened by the large stocks of Soviet short-range weapons, variously estimated at between 3,000 and 10,000, deployed with the forces of the Warsaw Pact. Quite what function they are meant to have is far from clear. Interdiction, NATO's purpose, is one, but the numbers seem excessive. Any other use, say for uttering threats against Western Europe or for bombardment in advance of conventional attack, would surely escalate. That is one reason why NATO has the strongest possible collective interest in negotiating on short-range weapons based in Europe. To the extent that its own deployment depends on the supposed superiority of Soviet conventional forces, it is natural that the talks on European nuclear and conventional forces should be linked — which is what the Soviet Union has been saying.

NATO's unwillingness to talk has several roots, of which the chief is that the Bush administration (to judge from Bush's speech last week at Texas A & M) has not yet made up its mind about Central Europe (and about much else besides). But others, notably a large proportion of the voting public in West Germany, have. Two calculations seem to matter most. First, Gorbachev's initiatives

on arms control seem to promise freedom from the threat of nuclear war. Second, as recent events in Poland and Hungary have shown, there is a chance that the states of Eastern Europe will now be much more free to act independently of Moscow than for the past 40 years. It is entirely reasonable that many people in West Germany should not wish to jeopardize these hopeful developments by seeing NATO's battlefield weapons modernized.

So what should NATO do? First, agree to negotiate about battlefield nuclear weapons, but insisting that the numbers of weapons allowed to each side are determined only in the light of the outcome of the Vienna negotiations on conventional forces. Among other things, negotiations could be used to explore Soviet views of the function of the Warsaw Pact's own battlefield weapons, perhaps then demonstrating to many in West Germany that life, while simpler than it used to be, is still fraught with danger. Second, agree that the introduction, planned for 1993, of the improved US Lance missiles, will be postponed until the negotiations are complete. (The Soviet Union was persuaded to withdraw its short-range SS-23 missiles from Europe on the grounds that their range might exceed the limit of 500 km stipulated by the INF treaty, but the planned Lance will be able to lob nuclear weapons over similar distances, which is why the Soviet governments believes it has been tricked.)

But is not to embark on negotiations on battlefield weapons to concede their abolition? That opinion, put about by Mr Henry Kissinger (who knows better) and apparently accepted by the Bush administration, is illogical. NATO should turn the problem around, setting out to define what conditions must be satisfied before battlefield nuclear weapons can be safely banished from Europe. One would no doubt be an even greater reduction of conventional forces than that now hoped for at Vienna. Another might be the maintenance of long-distance airborne nuclear weapons, which can substitute tactically for battlefield weapons; they are safer because their use can be more deliberately controlled and because they cannot be captured as easily, although they are admittedly more vulnerable.

That state of affairs would be a long way from Gorbachev's dream of a nuclear-free Europe. So long as there are aircraft carrying nuclear weapons, that will be so, but there are also the independent nuclear forces of Britain and France to be reckoned with. One of Mrs Margaret Thatcher's objections to West German misgivings about modernizing Lance centres on her belief that nuclear deterrence has kept Europe free from war for 40 years (which may be true) and that battlefield weapons contribute to deterrence (which is not the case, at least if NATO's theories are correct). But the British and French weapons are intended to be deterrent, and must be counted somewhere, preferably in the talks on strategic weapons now to be resumed. But here again, negotiating does not mean conceding. The difficulty, in this connection, is that the first need is for negotiations within NATO. Is the Bush administration ready for that ordeal?

Baltimore hearing ends without resolution

- O'Toole gives evidence
- Disputed paper upheld

Washington

A SECOND gruelling day of congressional investigation into the handling of allegations of error in a paper published in the journal *Cell* by Nobel laureate David Baltimore and four others ended on Tuesday 7 May with little sign of progress or direction.

The controversy over the paper has survived two university inquiries, a National Institutes of Health inquiry (recently reopened) and four congressional hearings, but still refuses to die, despite charges from the scientific community that the inquiry has turned into a witch-hunt. The Secret Service is to continue its forensic investigation of the laboratory notebooks of Thereza Imanishi-Kari, the principal author of the paper, for evidence of misconduct.

Compared to the confrontation between John Dingell, (Democrat, Michigan) chairman of the Energy and Commerce subcommittee on oversight and investigations, and David Baltimore the previous Thursday (see *Nature* 339, 83; 11 May 1989), the Tuesday hearing lacked high drama. But the day finished with an unexpected twist as sub-committee member Norman Lent (Republican, New York) suddenly revealed divisions in the subcommittee by attacking Margot O'Toole, the post-doctoral researcher who had complained about the paper soon after it was published in 1986.

The focus of the day's hearing was O'Toole and the two early attempts to deal with her complaints at Tufts University and Massachusetts Institute of Technology (MIT). Both inquiries were informal, convened long before the case came to be seen as a critical test of the ability of the scientific community to police itself.

At the hearing, O'Toole portrayed herself as a wronged whistleblower whose complaints, encouraged by university officials, meant that "I was left without a recommendation, I was left without a job and I was left without any support from anybody in the [immunological research] community".

Dingell, whose opening speech stressed the need to protect "whistleblowers who have the courage to come forward", sympathized. "O'Toole is sitting here with her career in ruins while others prosper mightily. Where's the justice in that?" he said, contrasting her fate with those she had criticized.

But minutes later, fellow subcommittee

member Lent attacked O'Toole's conduct by saying that he would not tolerate anyone in his own office who had acted as O'Toole had done, copying records belonging to another person and passing them to outsiders.

"I am very very positively impressed by the thoroughness and fairness with which . . . Dr O'Toole's complaints have been considered", said Lent.

Dingell left out the accolades as he searched for weaknesses in the way



No progress in Baltimore investigation.

inquiries had been conducted by the panellists from Tufts University and MIT who testified at the hearing. But even Dingell's toughest questions were thrown back by Henry Wortis of Tufts University.

Was Wortis disturbed by the finding that there was "no back up data for the fourth point on approximately six of the eight curves on figure one [of the *Cell* paper]?" "We're not talking about errors that have no meaning for science", said Wortis, "we're talking about whether the basic claim is true. And that is true."

How did corrections to the paper later ordered by the NIH committee reflect on the quality of Wortis's own examination of the paper, which concluded that no correction was necessary? NIH did not order "corrections" said Wortis, just "clarifications" that "left the central conclusion standing".

No progress was made on the case's central issue of when it is reasonable to ask to see a scientist's raw data and laboratory notebooks.

O'Toole's challenge, she accepts, is to the "state of the evidence at the time of publication" of the *Cell* paper. She still stands by her assertion that the "principal claim of the paper is not supported by the data". Her dispute is not of the traditional kind that sets one interpretation or set of results against another.

Neither of the first two investigatory panels examined the raw data, something O'Toole finds hard to understand. "Scientists should not regard their notebooks as personal diaries", she says, and requests for raw data should not imply a lack of trust. That view sets her apart from Baltimore, she says, who would not examine the data even "after I told him there was something wrong with it". If there had been more open access to data, O'Toole says the dispute would never have been dragged into the political arena. "This is the wrong forum", she said, "we're stuck here because we couldn't work out another one." O'Toole's views on data access have not found many supporters. As another of the subcommittee members Alex McMillan (Republican, N. Carolina) pointed out, testimony had already been given that a scientist's notes are there to support that person's work; everyone has a different style and some people substitute and add information. This view, he said, is very different from one that assumes that all notes have to be made "as if I am going to be challenged legally on their validity".

Although the issue shows no sign of resolution, and the possibility of legislation on the status of scientific data has not completely vanished, O'Toole's three-year spell of isolation as a whistleblower may be drawing to an end. She has hopes of another scientific job and an early return to the laboratory bench.

Alun Anderson

INDIA

Quick imports scheme

New Delhi

To speed imports of essential scientific equipment, the Indian government will now permit publicly funded research institutes and universities to import such equipment without paying customs duty.

Research institutes and universities will receive passbooks from the Department of Science and Technology entitling them to import equipment and consumables with self-certification. Eligible institutions can import equipment worth \$660,000 annually, and consumables worth half that much, by just showing the passbook to customs officials.

K.S. Jayaraman

AIDS

Industrial injury

Paris

A FRENCH nurse who developed AIDS after having pricked herself while dismantling transfusion apparatus from an AIDS sufferer has won a battle to define her illness as the result of an "industrial injury". This is the first case of its kind in France and, for the moment, means that she will receive state health benefits.

Peter Coles

CEC wants to double the money

Paris

THE Commission of the European Communities (CEC) has proposed that its European Community Action Scheme for Mobility of University Students (ERASMUS) should double its budget for the next three years. The proposal, expected to be debated at the Council of Ministers in December, would see the budget of 85 million ECU (1 European Currency Unit = \$1.1) for 1987–90 rise to 192 million ECU for 1990–93. ERASMUS was launched in 1987 with a budget of 10 million ECU, rising to 45 million ECU by 1989.

Its aim is to allow university students and teachers to spend periods of study abroad at universities in the 12 member states. But the success of the programme — applications exceed resources by a factor of three — has created problems. Students often find their grants inadequate and, in order to try to meet the demand, the length of the visits can be too short (between 3 months and a year).

In its first two years, ERASMUS enabled over 16,000 students to study at foreign universities within the Community. A goal of the Commission is that 10 per cent of the Community's 6.5 million students should profit from ERASMUS by 1993. Furthermore, it is hoped that the average length of stay should rise to six months and eventually to a full year. But language learning, not officially part of the ERASMUS programme, has proved to be a stumbling block. While other programmes within the CEC, such as the recently proposed *Lingua* programme, expressly deal with foreign-language learning, the Commission would like to see a budget set aside within ERASMUS to integrate language studies within the study period.

Peter Coles

BRAZIL

SONDA lifts off

São Paulo

THE Brazilian Air Force late last month launched successfully the fourth prototype of its 7.5-tonne sounding rocket SONDA IV. The rocket carries a 500-kilogram payload and, according to the Air Force's Institute for Space Activities it reached an apogee of 820 km. The SONDA series is the basis for the much bigger Satellite Launcher Vehicle (VLS), the rocket that will carry the Brazilian-built satellites in the next decade. VLS will have four SONDA IV motors as strap-on boosters.

The satellites are being developed by the civilian Institute for Space Research (INPE).

The Air Force also announced that a reduced-scale version of VLS would be launched on 16 May.

Ricardo Bonalume Neto

Does it work efficiently?

Chicago

ALTHOUGH the peer-review process steers the scientific machine, the 250 participants at the world's first international congress on peer review, held in Chicago last week, turned out to have only the slenderest of evidence that peer review works either efficiently or fairly. At one extreme is the machiavellian picture of peer review painted by Erdem Cantenkin of the University of Pittsburgh. In his talk he described peer review as a "club" with the "same individuals significantly involved in funding, publication and academic peer review processes". Conflicts of interest arise, he says, "because the funding and publication successes of members of this exclusive club are determined largely by other members of the club".

At the other extreme lies an exalted view of peer review as an impersonal truth-producing machine that maintains the objectivity of science and purges all error from the system. While some members of Congress may hope for this, some of the studies presented at the conference suggest that journal peer review may not so much eliminate errors as ensure they are published in someone else's journal. Papers rejected by one journal often make their way down a hierarchy of journals, acquiring only slight or no modification on the way, until they find acceptance.

Ignorance of the true state of peer review is unlikely to remain acceptable. The US National Institutes of Health decides how to spend \$7,000 million a year through peer review and as Peter Budetti, a member of a congressional subcommittee on health and the environment, pointed out, the public wants to know its money is well spent. Accountability for scientific activities has been delegated to scientists through peer review, Budetti said. That means that the peer review process itself must be open to public inspection lest Congress enforce more direct accountability.

Bias is the commonest form of complaint; especially amongst those refused publication or promotion by secret peer review. But a study by the *Journal of General Internal Medicine* shows reviewers and editors may not be as bad as they are painted. Eliminating bias by hiding the names and affiliations of authors and the identity of reviewers had little effect on editors' recommendations.

Publication bias does appear in a more subtle form. Francis Bacon described it succinctly in 1621 as the tendency of the human intellect to be "more moved and excited by affirmatives than by negatives". Several studies show positive results more likely to be published than negative ones. That practice may be stimulating but is dangerous: if clinical

trials are reported only when successful then ineffective drugs may reach the market-place. One solution is to register all clinical trials at inception so that negative results that never appear can still be traced.

Multiple publication appears to be thriving and peer review ineffective to stop it. Byron Bailey, editor of the *Archives of Otolaryngology — Head and Neck Surgery*, carried out an eight-year medical database survey and found that of 1,000 of his authors, 228 had published 938 duplicate articles. In a third of the cases there was no reference to the companion publication. Bailey also warned of "salami slicing", where many papers are produced when one would be appropriate, and sequential publishing, where data are added without changes in concepts.

Outright cheating is also unlikely to be picked up by peer review. Most journal editors argued that the integrity of the data was the responsibility of the research institution concerned.

Not all agreed because there are ways to check — random data audits of papers, conducted in the same spirit as income tax spot checks is one. But the recent Institute of Medicine report on *The responsible conduct of research in the health sciences* rejects data audits because they would rarely pick up irregularities and would have "a chilling effect" on creativity and independence of science.

Once fraudulent work gets into the literature it is not easy to get it out again. Paul Friedman of the University of California at San Diego found that journals were loth to publish retractions of work found fraudulent and many had no procedures for handling such requests. But Institute for Scientific Information director Eugene Garfield's study was more encouraging. He showed that citations of papers publicly exposed as fraudulent quickly dropped off as authors shunned the work.

Peer review has already gone too far for David Horrobin of the Efamol Research Institute. His look at the period from 1930 to the present shows, he says, that innovation has gradually been traded for quality control. Two effective psychiatric treatments — lithium for manic depression and monoamine oxidase inhibitors for depression — would never make it through the current peer review system.

His solution is *Medical Hypotheses*, the only medical journal fully devoted to ideas. Horrobin agrees that "many innovators are off the wall and pains in the neck" but he believes the world needs them.

Alun Anderson

The proceedings of "Guarding the guardians: Research on peer review" will appear in the *Journal of the American Medical Association* this summer.

Just one research council?

London

A RADICAL overhaul of the mechanisms for funding civil scientific research in Britain is needed, according to a review group set up by the Advisory Board for the Research Councils (ABRC), which advises the government on allocation of the science budget.

In a confidential report to the ABRC, the group recommends that the five research councils be replaced with one, to be called the National Research Council (NRC). With replacement of the ABRC by the NRC's governing council, the responsibility for all aspects of science spending would be vested in a single organization. The review group, chaired by Richard Morris, deputy chairman of the ABRC, has reported its conclusions to the ABRC, which is now waiting for responses from the research councils. If the ABRC accepts the report, it will then need the approval of the secretary of state. Implementing the reforms could take two years; as each council was set up by royal charter, legislation or further charters would be needed.

The problem which prompted the review was the overlap in responsibilities for the biological sciences. Biotechnology, for example, is funded through four research councils, and attempts at coordination have been unsuccessful, partly due to the problem of reconciling different styles of operation. The Biotechnology Advisory Group, set up in 1985 to improve co-ordination, is perceived as being substantially ineffective, says the report, and inter-council relationships are now so bad that suggestions by one are regarded with suspicion by others. Genetics is also singled out as an area in which there is significant overlap between councils, mainly the Science and Engineering Research Council (SERC) and the Medical Research Council (MRC).

The result is duplication of research, as well as neglect of research for which no council claims responsibility. Researchers in universities and industry are unsure which council to contact. And for research

problems that involve more than one council, a coordinated reaction is slow.

The review group rejected one possible solution: the formation of a council for non-medical biological sciences. This council would have to cooperate closely with SERC and the problem of cooperation between councils with different styles of operation would remain.

Instead it recommends formation of an NRC with six divisions (see diagram). The MRC would remain essentially as it is now, though some research might be transferred to the biology and environment division, or vice versa. The Economic and Social Research Council (ESRC) would also retain its current responsibilities.

Boundaries within one research council would be "more permeable" than those between the present research councils, says the report. Coordination of resources would be easier and there would be flexibility in allocation of resources. A single council would also mean significant savings in administration costs. And it would create a focal point for international links.

The governing council of the new NRC would have much greater control over the nation's science budget than the ABRC does at present. It would set priorities, allocate funds and be answerable only to the Secretary of State for Education and Science and to parliament. Although the ABRC has influence through its advice, it lacks the authority to ensure its recommendations are carried through, and is not accountable for the consequences of its advice.

On the NRC council would be the NRC director-general, the principal executive officer; a non-executive part-time chairman; the division directors; the chief scientist at the Cabinet Office; the chief executive of the Universities Funding Council; and eight independent members from industry or higher education. (These, as well as the chairman, would be appointed by the Secretary of State.) The council would have to produce a national strategy for science and engineering every five years and monitor progress against

Bottom of the spending league

Dublin

THE Irish government is running hard just to stay still in its support of science and technology. The Irish science and technology agency (Eolas) announced earlier this month that total spending in Ireland on research and development increased by 10 per cent between 1985 and the following year (the latest for which figures are available), but that the total remains a fixed proportion of gross domestic product at 0.9 per cent.

This puts Ireland firmly near the bottom of the research spending league, both in Europe and in a wider framework. Eolas notes that annual research and development spending in Ireland amounts to the equivalent of IR£47 per head, compared with IR£111 in Denmark, IR£205 in Britain and IR£360 in the United States.

Part of the trouble is that only 18 per cent of the government's science and technology budget is spent on research, but Eolas says that only 6.4 per cent of Irish manufacturing companies spend money on research and development of any kind, and that 17 foreign-owned companies account for two thirds of the country's spending in the field.

Mary Mulvihill

the plan.

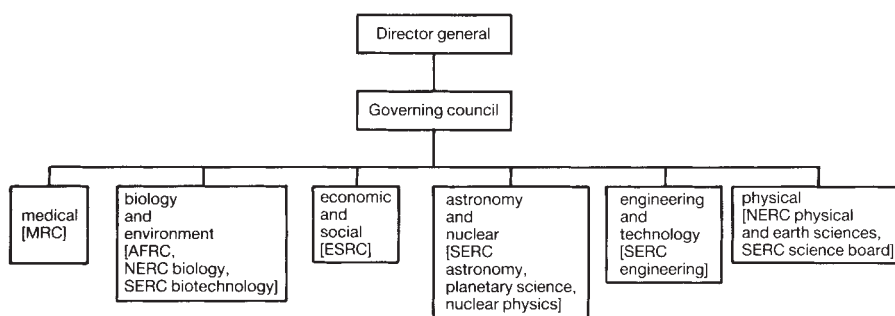
Senior positions in the NRC should be occupied by scientists with management training or experience, says the report, and the new council should encourage business schools to establish courses in management of research.

The idea of a single research council has been criticized in the past. The dangers are that it would be too large and remote from researchers. Policy errors in grant allocation would have more serious consequences than at present, and a reduction in the number of sources of funds might mean fewer opportunities for applicants. But the review group dismisses the criticisms. "Each of these potential dangers is avoidable", it says, but exactly how, it does not say.

As an immediate interim measure, pending formation of the NRC, the report recommends that the ABRC appoint two planning directors, for the biological and physical sciences, for drawing together the relevant elements of the AFRC (Agricultural and Food Research Council), NERC (Natural Environment Research Council), SERC and, if appropriate, the MRC to form the new divisions.

It also proposes giving influence within the NRC to individual 'coordinators', in areas where "high-powered individuals" are more effective than committees, such as coordination of biotechnology, development of policies for training research manpower and forging links with the European Community. **Christine McGourty**

National Research Council



Landsat to be bailed out?

Washington

THE US National Space Council headed by Vice-President Dan Quayle will recommend this week that President George Bush bail out the troubled Landsat satellite remote-sensing programme. The council — whose membership includes administration budget officials — voted unanimously last week to give EOSAT, the private company that operates the Landsat satellites, \$5 million to keep Landsats 4 and 5 running until the end of the year, and \$20 million to subsidize running costs for 1990.

The Landsat programme was nearly cancelled two months ago when the US National Oceanic and Atmospheric Administration (NOAA) ordered EOSAT to shut down the Landsat 4 and 5 satellites because there was no money to operate them. But without the Landsats, the United States would be forced to buy all non-defence satellite images from the French SPOT programme, and the Space Council came up with temporary funding that permitted NOAA to rescind its shut-down order two weeks after it was issued (see *Nature* 338, 365; 1989).

The \$5 million to be provided by the space council will come from the budgets of other government agencies that use the satellite images, such as the Department of Agriculture. Next year's budget is still being debated by Congress, but the \$20 million for 1990 is expected to be included in a budget amendment to be proposed by the administration.

The council also agreed last week that the launch of the new Landsat 6, scheduled for 1991, should proceed as planned. Under the agreement with EOSAT, NOAA pays for the development and launching of new satellites, but it now may have to take on part of the operating costs as well.

NOAA spun off management of the Landsat programme to EOSAT in 1985 in the hopes it would become a profit-making operation.

Although the government made a commitment to provide the operating costs of the two existing Landsat satellites, both have now exceeded their design lives, placing a burden on NOAA's perennially stretched budget.

The space council decision has been interpreted as an implicit admission by the administration that remote-sensing enterprises cannot be run as private companies that are expected to make a profit. The council is expected to recommend that President Bush make a "long-term commitment" to the Landsats, which may involve a reorganization of how the entire programme is managed. NOAA will continue to oversee the Landsats for the near-term, but that may change as a result of further deliberations.

Carol Ezzell

US-Japanese row continues

Tokyo

WHEN Ken Sakamura of Tokyo University set out in 1985 to redesign fundamentally the architecture of computers — both hardware and software — his dream was a new standard that would allow computers of all shapes and sizes to talk to one another throughout the world. Last week, that dream came down to earth as Sakamura took battle with the US trade representative, Carla Hills, who sees his independently developed computer system as a barrier to the sale of US products in Japan (see *Nature* 339, 88; 11 May 1989).

In a letter sent to Hills last week, Sakamura demands that all references to his TRON (The Real-time Operating-system Nucleus) project be removed from the 1989 National Trade Estimate Report on Foreign Trade Barriers. The report will be used to designate 'priority countries' for possible US trade sanctions by the end of this month.

The US trade office claims that the Center for Educational Computing (CEC), a foundation set up by Japan's Ministry of Education, Science and Culture (MESC) and the Ministry of International Trade and Industry (MITI) to select computers for high schools, has laid down specifications for the operating system of the computers that are favourable to TRON. And the US trade office fears that operating systems developed by US companies (such as MS-DOS and UNIX) will be shut out of MESC's lucrative \$6,900-million procurement of computers for Japan's schools.

Sakamura strongly denies these claims. Twelve companies have submitted experimental machines to CEC that use a TRON operating system. But the CEC specifications do not mention TRON. And the experimental computer submitted by NEC Corporation of Japan can operate on MS-DOS as well as a TRON operating system, according to Sakamura in his letter to Hills.

Japanese subsidiaries of the US companies IBM and Unisys Ltd. are among those which have submitted experimental machines to CEC, and CEC's hardware specifications match those of an IBM personal computer with a 16-bit microprocessor made by the US company Intel. Sakamura thus says that the US trade office's contention that "no US manufacturer is in a position to sell TRON-based personal computers" is "incorrect". And he adds that he made the basic design of TRON architecture freely available to all companies, Japanese and foreign alike, to prevent the development of "proprietary" (and incompatible) technology.

Sakamura openly admits he is a "dreamer". And some of his ideas do seem to verge on fantasy. A promotion

video for TRON declares that in the TRON "intelligent house" of the future TRON sensors embedded in the walls will open and shut the windows like a "king's servant" to create the kind of natural breeze that human beings like. And in "TRON City", microprocessors in the road will automatically detect a child running into the street and command TRON cars to grind to a halt and avoid collision.

But such is the way to promote new technology in Japan and the huge consortium of companies that are now backing TRON have their feet firmly planted on the ground. Following a decision by Motorola and Intel of the United States not to license their latest 32-bit microprocessors to Japanese companies, three Japanese electronics giants — Hitachi, Fujitsu, and Mitsubishi Electric — banded together in 1987 to develop 32-bit TRON microprocessors. Membership of the TRON Association, a grouping of companies set up last year to promote TRON, has swelled to 125, including Motorola, Intel and more than 20 other European and US companies.

According to TRON advocates, the 32-bit microprocessors from Motorola and Intel are like old factories with extensions built on — their ability to process data is slowed down by their need to maintain compatibility with old technology. But TRON 32-bit microprocessors, samples of which are now available, have been developed hand-in-hand with their operating systems.

TRON operating systems are also designed to handle the Chinese characters (*kanji*) of the Japanese language. It takes twice as many bits per character to encode the thousands of *kanji* in Japanese as it does to encode roman letters or numbers. And until recently, personal computers have not been particularly adept at Japanese word processing.

But will TRON penetrate the world market? In the United States and Europe, where there is already a considerable infrastructure of computer hardware and software, it seems unlikely that users will rip out and replace their present systems. But some of the TRON 32-bit microprocessors soon to be launched on the market are compatible with the widely used UNIX operating system developed by AT&T.

It is in Japan that TRON has the best chance of making inroads. Personal computers are conspicuous by their absence in Japanese offices, homes and schools. Computer networks are also in their infancy. There is thus an open and waiting market when the first commercial TRON computers come available in 1990.

David Swinbanks

More money available

Sydney

A SURPRISE boost for Australian science and technology was delivered by the government last week in a A\$1,000 million increase in funding, tax cuts and scholarships. Under the banner "science creates wealth", the government hopes that the package, to be spent over the next six years, will encourage investment in research and development by industry and entice those entering science to make it a career.

Australia ranks fifteenth in the world in science spending by the private sector. To improve this rating, the government has extended its 150 per cent tax deduction on research undertaken by industry until June 1993. After that, a 125 per cent deduction will operate for a further two years, costing the government an expected A\$640 million.

Much of the new budget emphasizes increasing public awareness of science. Professor Ralph Slatyer, dean of the Research School of Biological Sciences at the Australian National University in Canberra, will take up the newly created position of Chief Scientist, advising the

Prime Minister, Bob Hawke, and the Minister for Science, Barry Jones. Jones has increased his own status by becoming the Minister Assisting the Prime Minister on Science and Technology. Jones will also become the deputy chairperson on the new Prime Minister's Science Council, which will include representatives of the ministries associated with science and technology as well as members of the science and business communities.

To raise international awareness of Australian science, the government has created the Australia Prize, worth A\$250,000 tax-free annually, to go to an international scientist or group of scientists for "excellence in promoting the welfare of peoples of the world". Other prizes include two MacFarlane Burnet Fellowships, each worth A\$230,000, for



Barry Jones — up the ladder again.

medical scientists working overseas who wish to return to Australia.

Higher education was a big winner in the budget, receiving A\$977.9 million over the next five years for research and postgraduate awards. From 1990, all Commonwealth postgraduate research awards will provide between A\$12,734 and A\$16,433 tax free, double what students received in 1986.

The Commonwealth Scientific and Industrial Research Organisation (CSIRO) remains the loser in the budget. In a week in which 500 staff were told they would lose their jobs or be transferred to other departments, the organization received only A\$14 million extra for 1989–90, rising to A\$19 million for each of the next four years.

But CSIRO needs A\$250 million merely to cover the budget cuts over the past five years. The budget loss for this coming year alone is A\$40 million because of government cuts, a shortfall in private sector funding and a A\$14.2 million accounting miscalculation. As Jones put it, "the incentive is clear for CSIRO to boost its revenue and research capacity by actively seeking a major industry R&D expenditure".

Tania Ewing

Efforts abandoned in Japan

Tokyo

NEWS of cold fusion has caused wild excitement in Japanese laboratories, but so far all attempts to replicate the work have failed. Some researchers have already abandoned their efforts and, whatever might be said in US congressional hearings, there are no government plans to support a national research project.

Within days of the announcement by Utah research groups that they had achieved fusion in a flask, a broad-based effort to replicate the experiments began in dozens of laboratories around the country. Japanese scientists took to the research with the same unbridled enthusiasm they showed following the discovery of high-temperature superconductors two years ago. But this time all there is to show for their efforts is one false alarm.

On 1 April, Japanese newspapers reported that Noboru Oyama of Tokyo University of Agriculture and Technology had observed large amounts of heat and gamma rays when an electric current was passed between palladium and platinum electrodes in heavy water.

But Oyama says the press reported only the "positive" aspects of his results. Subsequent experiments in collaboration with researchers at the Japan Atomic Energy Research Institute have failed to detect emission of neutrons, tritium or helium-4. And calorimetry experiments will not be completed for several months, Oyama says.

Other groups with expertise in nuclear fusion and neutron detection report similar negative results. And a group at Hokkaido University headed by Hiroshi Ohashi announced a few weeks ago that it was abandoning its experiments because of failure to detect any products of nuclear fusion.

Nevertheless, interest in government and industry remains high because Japan, which depends on imports for most of its energy, would stand to gain enormously if cold fusion turned out to be a practical source of energy. And several symposia on cold fusion will be held over the coming weeks.

But, in contrast to the case of high-temperature superconductors, no government ministry or agency has established or plans to establish a 'study group' (*kenkyukai*) of industrialists and academics to promote national research on cold fusion.

Cold fusion still needs to be proved says Shigeharu Kato, deputy director of the Technology Development Division of the Science and Technology Agency's Atomic Energy Bureau, an organization that oversees Japan's research development of nuclear fusion.

David Swinbanks

HIGHER EDUCATION

Bonn courts young and females

Hamburg

WEST Germany is facing an education crisis unless young university faculty members are persuaded to keep their posts until an expected wave of retirements in the late 1990s. To head off the problem, West German Education Minister Jürgen Möllemann (Free Democrat) has announced a plan to invest DM800 million annually in salaries and research support for young researchers. So far, two *Länder* have voiced support for the plan, and Chancellor Helmut Kohl (Christian Democrat) has agreed in private that "something must be done", said a spokesman for the Education Ministry. Möllemann also plans to make funds available specifically for women researchers.

According to current plans, 60 per cent of the new money would come from the federal government and 40 per cent from the *Länder*. Achieving consensus on the plan could take six months or more and the amount of money could be reduced dramatically if federal and *Länder* finance ministers tighten the pursestrings.

But education has been a popular cause in West Germany lately. A programme to provide DM2,100 million in additional support for university teaching in overcrowded fields like business studies and information sciences came into effect earlier this year.

Steven Dickman

Who will save the planet?

Washington & London

THE Bush administration last week received a commitment from countries meeting in Geneva on global climate change to hold a workshop in Washington in October to prepare the ground for a framework convention.

This display of enthusiasm for action on climate change contrasts sharply with an incident earlier in the week, when the White House admitted revising a government scientist's testimony before Congress to present a less gloomy picture of global warming.

The Bush administration was severely criticized last week for altering the testimony of National Aeronautics and Space Administration scientist James Hansen to make his conclusions about greenhouse warming appear less certain (see *Nature* 339, 84; 11 May 1989). The criticism was especially pointed in light of Bush's campaign rhetoric promising to tackle the problem of greenhouse warming.

At a hastily arranged press conference last Friday, Environmental Protection Agency (EPA) administrator William Reilly denied that the commitment to hold a workshop was a change in direction for the administration, and reiterated Bush's commitment to solving environmental problems.

The October workshop will address five topics: legal and institutional issues, economic issues, questions of public education, technology transfer and the role of international banks. Each nation attending will prepare papers addressing these topics, which will be used to form a consensus that can be presented to the International Panel on Climate Change (IPCC) in early 1990. By the end of that year, IPCC should be able to move forward with attempts to produce a framework convention that would form an umbrella for protocols to address climate problems.

Reilly said the administration was especially anxious to treat seriously the concern that strict conservation measures may stifle economic growth in developing nations. He denied that the United States is trying to wrest from any other country world leadership in addressing climate change. He maintained that the United States takes global warming seriously, and would encourage others to do so, but would not trivialize the problem by "responding to fashion".

"There is a consensus in the administration that global warming presents a threat to the country and the economy", said Reilly, although he admitted that within the administration there were some differences over how great the threat is. He insisted that any effective steps to stop global environmental damage will have to be taken in concert by all nations.

The British government is also keen to be seen at the forefront of international activity to tackle the problem of climate change. Britain's environment minister Lord Caithness will attend a meeting of the governing council of the UN Environment Programme (UNEP) in Nairobi this week, where he will report on the outcome of a seminar on climate change convened by the Prime Minister, Mrs Margaret Thatcher, held at her house at 10 Downing Street last month.

Caithness is also expected to endorse the formation of an international conven-

CHINA

Student protests bed down

London

THE hasty transfer last Monday of China's official welcome to Mr Mikhail Gorbachev from Beijing's Tiananmen Square to Beijing airport is a telling measure of the diffidence of the authorities in dealing with the demonstrating students who had occupied the square. But it is too soon to know how the students' demands for openness and democratization along Gorbachev lines will be dealt with.

The demonstrations began a month ago, after the death on 15 April of Hu Yaobang, the reformist general-secretary of the Chinese Communist party, who was replaced in January 1987 after a previous wave of student unrest. Many students felt that Hu had been unfairly ousted, and that he had been let down by insufficiently resolute students and intellectuals. Among the immediate causes of the demonstrations was the failure of the central committee's official obituary of Hu even to mention his disgrace in 1987.

On 18 April, a student meeting in Tiananmen Square formulated a seven-point demand to the authorities, calling for a "reassessment" of Hu Yaobang's merits and faults, the affirmation of "democracy, freedom, magnanimity and harmony", redress for those wronged in the "anti-spiritual pollution" and "anti-liberalization" campaigns of 1987, an end to corruption among party officials and to press censorship, more money for education and higher salaries for intellectuals.

The same day, Professor Fang Lizhi, the astrophysicist, who lost his university post in the 1987 purge for having allegedly been a harmful political influence on his students, told an Israeli radio reporter that the students had taken the opportunity of Hu's funeral to advocate democracy and freedom.

The authorities were at first unwilling to respond to the students' demands. The new 'student solidarity associations' at

tion on climate change that would be followed up with specific regulations, in the same way the Montreal Protocol for the protection of the ozone layer builds on the Vienna Convention.

He plans to urge that UNEP, which joined with the World Meteorological Organisation to form IPCC, should be strengthened by giving it more resources and greater powers. Britain has just doubled its contribution to UNEP, from £1.25 million to £3 million, he said, and other countries should do likewise.

The governing council will also consider how the IPCC can attract greater participation among developing countries.

Joseph Palca & Christine McGourty

Beijing's universities and higher colleges were denounced as illegal, reports of students injured in clashes with the police were officially denied and (according to a Hong Kong source) the Chinese media were instructed to ignore the students' demands.

A further call for dialogue, made on 21 April by a 100,000-strong rally, was ignored, whereupon the students went on strike and pasted up around the universities lists of important and well-paid jobs held by the relatives of party leaders. (One was the post of director of the State Commission for Science and Technology, which is held by Deng Nanm, daughter of Deng Xiaoping.)

Dialogue was joined on 29 April, when a government delegation held "frank and sincere" talks with 45 students drawn from 16 Beijing universities and higher colleges (but not, it seems, from the leadership of the new unofficial student unions). The government side afterwards claimed that the wishes of the "broad masses" of the students for the most part coincide with its own, and said that the government had already increased the education budget and was taking measures to rectify its "mistake" in paying intellectuals so little.

Since the early days of this month, mass demonstrations have abated, but petitions for swifter and more effective dialogue continue, while the mood of student criticism and protest has spread to all parts of China, including the ethnic minority areas.

Efforts by the authorities to win the students' confidence by assuring them that no students will be punished for expressing their opinions, provided that they have not broken the law, proved ineffective in ending the protests — possibly because these assurances have been accompanied by condemnation of the new student unions and the demonstrations they organize as "illegal".

Vera Rich

Fire strikes Jackson Laboratory

Washington

A FIRE last week at the Jackson Laboratory in Bar Harbor, Maine, severely damaged the laboratory's production facility, killing some 500,000 mice and putting a halt to shipment of numerous unique strains of mice. By the week's end, officials had determined that none of the foundation stock had been lost, and Kenneth Paigen, incoming director of the laboratory, was bravely predicting that shipments would resume within 60 days. But it will be many months, if not years, before pre-fire conditions can be restored.

"This is a catastrophe", says immunologist Gerald Callahan of the University of Colorado, Colorado Springs, a common reaction to news of the fire. Although not the largest supplier of mice by volume, Jackson Laboratory is not only the sole source of certain mouse strains, but JAX mice are famous for their genetic purity and freedom from disease. The unique strains C57BL/6J-db and C57BL/KsJ-db have become extremely popular in diabetic research. Other strains in high demand are a dystrophic mutant 129/ReJ-dy and a lymphoproliferation mutant MRL/MpJ-lpr used for studies of immune disorders.

The laboratory distributes approximat-

ely 2-3 million mice a year drawn from 1,700 different strains. The \$10-12 million in revenue from mouse sales is used to support research at the laboratory.

The fire started shortly after one in the afternoon. Four workmen were remodeling a room in the one-storey production facility when a volatile solvent for an adhesive they were using caught fire. Twenty-four hours later the fire was still smouldering. None of the research buildings on campus was damaged, and the production facilities for the mice used by Jackson researchers will not be affected by the fire. Foundation stocks exist either as live breeding pairs or frozen embryos.

For some at the Bar Harbor facility, last week's fire brought a chilling sense of *déjà vu*. In 1947, a fire completely razed the laboratory — as well as part of the island community where it is located — destroying all mouse stocks. The present laboratory's genetic stocks were rebuilt at that time from donations from laboratories all over the world.

Paigen, who will take over as director of the laboratory later this year, was visiting the laboratory last week, and had left for the airport to return to the University of California at Berkeley where he is on the

faculty when news of the fire brought him racing back to the laboratory.

In addition to the good news about the foundation stocks, Paigen says that at least a portion of the production facility was protected from total devastation, and may be restored. In the meantime, the laboratory plans to look for temporary quarters off campus. Other breeders, notable Harlan Sprague Dawley and Taconic, have offered to pick up some of the production capacity until Jackson Laboratory can get back on its feet. Paigen says he hopes that shipments can resume in 30-60 days, although he admits "there may only be two mice on that truck". **Joseph Palca**

HDTV

Europeans unperturbed

Paris

HIGH-definition television (HDTV) is a new arena for intense competition between US, Japanese and European manufacturers, but the three-way tug-of-war over norms looks set to end in a stalemate. As the Japan Broadcasting Corporation (NHK) gears up to begin broadcasting daily high-definition pictures via its BS-2 satellite next month, US manufacturers have decided to follow the Europeans by pooling capital and seeking government aid.

On Tuesday 9 May, the American Electronics Association, a trade group of more than 3,500 companies, asked Congress for \$1,350 million in grants and loans to give the US electronics industry sufficient muscle to win a share of what seems likely to be a lucrative market, with valuable spin-off in semiconductor technology (see *Nature* 338, 106; 1989). Industry-wide collaborative research has long been practised in Japan and provided the model for Europe's EUREKA programme, which chose HDTV as one of its first projects in June 1986.

But the dream of a multi-million dollar world market for HDTV shows every sign of evaporating now that the United States no longer intends to adopt the Japanese Hi-Vision norm of 1,125 lines at 60 Hz. Both US and European designers have chosen a simple doubling of existing standards, giving 1,050 lines in the US and 1,250 lines in Europe, much to the chagrin of Japanese manufacturers who had hoped to adopt a universal norm.

Meanwhile, more than 30 European manufacturers, led by Thomson, Thorn-EMI, Bosch and Philips are confident that their system is the best. The \$220,000-million EUREKA project already has operational prototype HDTV receivers using the TDF-1 broadcasting satellite launched last year and manufacturers could begin production in 1990. The European Commission's JESSI programme, launched in February to develop new-generation semiconductors (see *Nature* 337, 682; 1989), should help. **Peter Coles**

NUCLEAR POWER

Indian energy agency blasted

New Delhi

POOR planning and management have resulted in construction delays, lost revenue and reduced capacity for India's nuclear power industry according to two government reports released two weeks ago. The Department of Atomic Energy (DAE) was also accused of slipping up on quality control by accepting substandard pipes meant to carry toxic nuclear wastes from the Narora Atomic Power Plant commissioned last March. But DAE says that delays and cost escalations are an unavoidable result of greater self-reliance and use of indigenous nuclear technology.

The parliamentary public accounts committee, which keeps an eye on financial mismanagement in government projects, criticized DAE for its management of its heavy-water plants and the two reactors of the Madras Atomic Power Plant (MAPP). The committee claimed that DAE had not carried out an adequate site evaluation nor did the agency make a realistic assessment of the industrial infrastructure. As a result, production targets constantly slipped. MAPP-1 was eight-and-half years behind schedule and MAPP-2 took nine-and-half years longer than expected to complete. The cost of the project rose from Rs1,320 million to Rs2,460 million.

The accounts committee said poor planning forced one of the Madras reactors to

remain idle for 16 months for lack of heavy water, resulting in a loss in revenue of Rs564 million. The committee also expressed concern over the poor performance of the two Madras reactors, one of which has been shut down because of a heavy-water leak from a coolant channel.

DAE's heavy-water plants were criticized for their inefficiency and high cost of production. The plant at Tuticorin is India's best and, according to the committee, its average production was only 20 per cent of installed capacity. It produces heavy water at a cost of Rs13,800 per kilogram, more than ten times the estimate.

A separate report from the comptroller and auditor general issued last week said that DAE's plant in Baroda produced heavy water at Rs6,846 per kilogram against the initial estimate of Rs478. Both plants were built by the French company Gelpira. The Baroda plant was 53 months behind schedule and DAE over-spent Rs180 million on its construction.

The auditor's report also blamed DAE for poor quality control. According to the report, DAE accepted 1,800 metres of "deeply pitted and poor quality" carbon steel pipes for transporting liquid wastes from the Narora plant. The partially laid pipeline had to be dug out and replaced with stainless steel pipes after the defects were revealed. **K.S. Jayaraman**

How to define entropy

SIR—A suitable definition of entropy was not possible at the time of publication of the 'E' section of the first edition of the *Oxford English Dictionary*, because quantum theory had not yet been discovered. It is sad that the new edition of the *OED*¹ reviewed in *Nature* by Stephen Jay Gould², does not fill the gap in the definition, except perhaps as a branch of information theory. It seems that no dictionary has advanced beyond the age of steam.

The problem in constructing a contemporary definition is usually circumvented by defining entropy in terms of its traditional properties. A good definition³ of this type is: "(Phys.) Measure of the unavailability of a system's thermal energy for conversion into external work; measure of the degradation or disorganization of the universe". A specialist, however, would ask for a definition applicable to an isolated system with a more or less well-defined energy and number of particles.

Because entropy is not really a classical quantity, we must build quantum mechanics into the definition. Landau and Lifshitz⁴ put it well: "Only the concept of the number of discrete quantum states, which necessarily involves a non-zero quantum constant, enables us ... to give an unambiguous definition of the entropy".

It suffices to define entropy as: the logarithm of the number of quantum states accessible to a system. This sentence is best known by word of mouth, perhaps starting with the lectures of Tolman and Oppenheimer, but it has appeared in the occasional textbook⁵. The quantity thus defined has all the necessary properties. It can be a terrifying experience to count the

states of a large system in some yet-to-be-specified energy range, but we rarely have to calculate from the definition. We work from the temperature and the chemical potential, which are the derivatives of entropy with respect to energy and number of particles, to the Gibbs or Boltzmann distributions, from which the entropy is then readily calculated.

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Not abandoned

SIR—I was surprised to learn in the slightly misleading News and Views article by Marek Abramowicz and George Ellis (*Nature* **337**, 411; 1989) that a feature of the Venice conference on cosmology and philosophy had been my abandonment of the final anthropic principle. In fact, my talk was concerned primarily with other subjects. But the documented discussion following other speakers reveals that the scientific basis of this proposal was presented and defended. It is particularly interesting in view of recent ideas about information-processing and complexity. For a truer picture, I refer the interested reader to the conference proceedings, which will be published by Cambridge University Press under the editorship of U. Curi.

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Peer review?

SIR—We recently learned of the claim that successful experiments have been carried out in a laboratory demonstrating that nuclear fusion is possible.

I would hope that *Nature* will feel able to carry out an investigation of this claim, and on the basis of past experience I suggest that the investigating team be made up of (1) a journalist with a scientific background, preferably in a subject far removed from nuclear physics, (2) a professional conjuror, (3) an expert in scientific fraud.

Such a team, with their undoubted expertise, will surely be well-qualified to determine the validity of the claim.

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Moral responsibility

SIR—We agree with Jamie Love (*Nature* **335**, 758; 1988) on the moral responsibility of scientists involved in military research: such a position has been taken by many leading scientists in the past (Einstein for example), but the problem remains because of a minority of scientists and engineers who are still working on projects with possible military application. It is our duty to encourage them to change their minds.

The whole international community should help those who want to change from military to civil research, if they get into trouble for this reason: the greater the risk from this choice, the greater the merit of a scientist who acts in this way and the greater the help he should receive from the community.

This is why many scientists, including Nobel prizewinners, are now supporting

Mordechai Vanunu, the Israeli nuclear engineer who was kidnapped in Italy by the Israeli secret service and sentenced in Israel to 18 years in jail because he left his job at the nuclear plant of Dimona, which produces weapons-grade plutonium, and revealed the existence of at least 50 Israeli nuclear warheads.

We suggest that all peace-loving scientists should support Vanunu by sending letters to the Israeli court or to embassies, by collecting signatures or by acting in any other way they think could be useful.

The release of Vanunu would be welcomed by all those now considering the possibility of ceasing to be "techno-accomplices to murder".

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Selfish academics?

SIR—The recent correspondence in *Nature* regarding the necessity or not of reprints and reprint requests has perhaps best illustrated academic selfishness rather than any other point. There are scientists who feel that it is more efficient for themselves to send multiple reprint requests rather than to visit the library and make a single photocopy for their own use, thereby throwing the burden of time and cost onto the author rather than the requester. Similarly, those antagonistic to reprint requests (for reasons other than impecuniness) are selfishly denying the genuine requests among those that they have received.

However, the principle may be obscured by the influence of human nature. In my experience, scientists who need or want to answer reprint requests will continue to do so, although often for reasons that are closer to personal advertising than scientific altruism. Conversely, scientists who request reprints are diluted beyond those with genuine purpose by others with either inherent laziness or insecurity. Scientists in most Western countries should be able to fulfill their needs through modern library facilities (including electronic searching and inter-library loans), although reprint requests from scientists in relevant institutions not so fortunate as to have these facilities should still be met where possible with politeness and encouragement (that is, a reprint or photocopy of the article in question).

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With dosimeter in the sarcophagus

Yuri Kanin

On the third anniversary of the Chernobyl reactor accident, *Nature's* Moscow correspondent carried a dosimeter from Kiev to Prip'yat and into the Chernobyl sarcophagus.

Chernobyl, Ukraine

It was my third trip to this region in the three years since the sadly memorable disaster at the Chernobyl nuclear plant. The previous two, however, were one-day visits. This time, I lived for four days alongside those who are now working there, enjoyed "most favoured status" in meeting those I wanted to see and was the first correspondent of a foreign journal to have been inside the No.4 unit, now known all over the world as the sarcophagus. What follow are my impressions, sharpened by the clickings of the portable dosimeter with which I was provided.

Newspapers and television in some Soviet cities now regularly report on the radiation situation. Not so long ago, the press carried maps showing radioactive zones in Byelorussia, the Ukraine and the Russian Federation. In Kiev, with a population of 2.5 million and about 180 km from Chernobyl, the local paper had such a map of the city. But, as a natural consequence of the habit formed by the veil of secrecy, one's own dosimeter is more reliable.

The Ukrainian Republic hydrometeorological board reports that the radiation background in Kiev is 0.014–0.023 milliroentgen an hour. In the capital of the zone [Chernobyl]: level of gamma background 0.04–0.17 mr per hour.

You reach Chernobyl, capital of the zone, from Kiev by car. You can proceed further, to the former town of Prip'yat and the nuclear plant, only by using vehicles doomed to operate in the zone alone.

The check-points on the road are where they were during my previous visits: on the border of the 30-km zone and when entering Chernobyl, which is 18 km from the nuclear plant by road and 15 km as the crow flies. The traffic is not as heavy as in 1986, but vehicles are still separated into 'clean' and 'contaminated'. The radiological inspection and decontamination points are still there.

Chernobyl, with almost 800 years of history, had a pre-disaster population of 18,300. Now, it houses 6,000 people on a scheduled basis: 15 days in the zone and 15 days with their families outside. The buildings are repeatedly decontaminated. *Kombinat* workers have permanent dwellings in Kiev, Chernigov and villages outside the zone.

Although radioactive objects have been

decontaminated and removed and short-lived isotopes have disappeared, the 'caesium period' has now set in. (Long-lived caesium-137 and caesium-134 will be the chief contaminating isotopes for a considerable time.)

The town will, of course, remain. My firm belief is that it must become the centre of an international research site. Many of those to whom I have talked in Moscow, Chernobyl, Prip'yat and at the nuclear station are coming round to the idea. There is undoubted interest abroad.

But some people have already returned to live in villages in the 30-km zone, happy to be at their hearths again. About 1,000 people are now living with elevated radioactivity. They are supplied with uncontaminated food and regarded as brave people defying an invisible threat. But why do not the authorities exercise their authority? Most of them may be old people who have found it difficult to put down roots in new places, and the levels of radioactivity in the villages may be low. But people do not stay home all the time; they go to the woods to pick mushrooms, they tend grazing cattle and many of them drink locally produced milk. Grandchildren are already paying visits, to take a country holiday and breathe 'fresh air'. The zone is no place for permanent living.

Once-beautiful ghost-town Prip'yat: 0.2–1.6 mr per hour.

The former town of Prip'yat, inhabited by power workers, is 3km away from the nuclear plant. The road to it has two more check-points (with an obligatory search on the way back, to prevent looting).

It took two hours for Prip'yat's population of 45,000 to evacuate in April 1986. Now there are no permanent inhabitants, nor will there be any.

A well laid-out town, with neat dwellings, social and everyday areas: through the darkened windows, one sees empty rooms from which everything has been taken away and buried. There are huge shop windows with no goods on the shelves. There is a comfortable stadium which was to have staged its first football match four days after the catastrophe. And there are new signs: the famous blue spruce trees are blue only at the top, with the rest of their needles green as in pine trees, but half as long again.

Yet a sports complex, with a 25-m swimming pool, an exercise hall and a sauna,

enjoys great popularity among those with access to Prip'yat. The town, which accommodates only mobile teams, has a small communal services contingent and two enterprises — 'Kompleks' and 'Spetsatom'.

Kompleks director Vitaly Starodumov says that the decontamination of the most radioactive land around the plant is almost over. "There has been no spread of radioactivity beyond the boundaries established at the time of the disaster." There is virtually no wind transport of radioactive dust because of the work done in the removal of topsoil, its recultivation and chemical and biological treatment. Starodumov says that the air activity in Prip'yat is the same as in large industrial cities (10^{-16} per litre), but there are houses and areas where the contamination by heavy long-lived elements is still high (0.2 Ci km^{-2} on the average).

At the outset, radioactive waste was buried in provisional burial grounds, which are monitored by a network of more than 500 boreholes located, on the recommendations of hydrologists, where underground water flows converge. Every 15 days, a group of workers takes measurements which have not yet revealed changes in the isotope content.

Starodumov says that Kompleks is now creating a protective buffer zone of forest belts around the nuclear plant and Prip'yat. It is also trying out a novel decontamination technology in a programme called Poligon. Three years ago, when the activity was high and the work dangerous, decontamination meant spraying, the removal of topsoil or both. Now the objective is to use electrochemical, acid, alkali and high-temperature and polymer treatments to process the waste into a compact mass. Then, extending the network of burial grounds can be avoided while the emplacement of compacted waste in fixed storage will be much more reliable.

I visited one of these burial grounds being modernized by automation for highly active waste (not less than 5 per hour). It is surrounded by a concrete wall 8.5 m high and up to 2.5 m wide. Inside, my dosimeter registered 3.2 mr per hour of gamma radiation and 0.3 mr per hour in the operator's room. Vehicles carrying waste (the driver's cab clad with lead and provided with a filter and ventilation unit) would drive up to a telescopic robot monitoring the activity, the operator would judge from his instruments into which section the cargo should be put and

a remote-controlled tower crane would handle it.

Rapid Emergency Management Service (record the address of this service just in case): Spetsatom Association, city of Prip'yat, the Ukraine.

Pripyat's other enterprise — called 'Spetsatom' — is the only one of its kind. Located on the site of a former tape-recorder factory, its workshop has a mock-up of a Beloyarskaya nuclear-plant module. Surprisingly, my dosimeter did not respond in any way. These fellows know their job!

"Not a single firm in the world has the same experience of emergency operations complicated by radioactivity, or comparable equipment to do the job, or people with such training", says Yuri Samolenko, president of the Spetsatom Association. (He received the title of Hero of Socialist Labour, the highest civilian decoration in the Soviet Union, for his part in the aftermath of the Chernobyl accident). "We are ready to respond quickly to a signal from any part of the world afflicted by a natural calamity or a major industrial accident."

Emergency groups from the Spetsatom Association have already demonstrated their capabilities in Armenia. In just 48 hours last December, they airlifted 18 units of heavy equipment (a crane with a load-lifting capacity of 200 tons among them) to the earthquake-stricken city of Leninakan. Spetsatom's performance, the giant Soviet-built Antei and Ruslan transport aircraft used in the operation and the organisation of the emergency operations even aroused the interest of visiting officials of the US Federal Emergency Management Agency.

The Spetsatom Association was founded a year ago as an emergency service of the USSR Ministry of Atomic Energy. It is affiliated to an interdepartmental research and development agency uniting more than 40 Soviet institutions and organizations that develop, test and produce robotized equipment and develop methods for its use.

Next July, Spetsatom plans to test in the Chernobyl zone an installation for soil-decontamination using infra-sound. It can separate, fraction by fraction, radioactive particles with sizes ranging from 20 to 400 μm . One installation can decontaminate 100 tons of soil an hour (or, in one year, could treat soil to a depth of 0.2 m over an area of 100 km^2).

But Spetsatom's chief task is to cope with emergencies at nuclear power plants and to decommission generating units at the end of their life. In the workshops, I saw a full-scale model of part of a nuclear reactor installed on a jig with a gantry crane (used to transport robots). It represents the reactor which served for 25 years at nominal capacity at the Beloyarsk nuclear power plant.

The reactor has been shut down and will be dismantled by Spetsatom. Samolenko hopes the new techniques will make it possible to reduce the time for decommissioning from between 8 and 12 years to about 4 years.

Two hours inside the three-year old sarcophagus.

The sarcophagus containing the crippled reactor, although a large and impressive structure, is no tourist site, you must understand. But many parts of it are now readily accessible.

An expert group from the Moscow-based Kurchatov Institute of Atomic Energy has been here drilling holes into the bowels of the destroyed reactor. Why should anything of this sort be done?

Rumours rife in Kiev have it that the crane towering over the sarcophagus is working once again, dismantling its roof, and that the operation may lead to new radioactive emissions. It is also alleged that the red-hot fuel is burning its way through into the ground. Some even say that a critical mass may develop and lead to a nuclear explosion. Why all these rumours? Radiophobia or justified fear?

Igor Kambulov, head of the Kurchatov Institute group, says that the objective is partly research and partly the enhancement of the safety of the structure. The group plans to tell by drilling the exact location of the fuel mass. "We have found that its state is stable and sucritical by a wide margin", so that a spontaneous chain reaction is highly improbable. But, to enhance the degree of subcriticality so as to preclude a chain reaction under all conceivable circumstances, the group has been inserting further neutron absorbers. "The fuel mass is being cooled by air from below, from the bubbler. The maximum temperature of the fuel is 150° C and it is falling steadily."

Kambulov says that the fuel is not moving and that the supporting structure is not sinking. The group is monitoring the structural elements of the sarcophagus, reinforcing those in doubt, and also using special compounds to bond aerosols and so prevent the transfer of radioactivity.

"And now about the crane. We used it, indeed", says Kambulov, "but for decontaminating the engine-room." Three years ago, the top priority was the sealing of the crippled reactor; the engine-room, which was seriously damaged and contaminated, was to have come next. Now, Kambulov says, "we have removed everything that could be removed from there". His group has also poured decontamination compounds and concrete into the engine-room and built a new roof over it.

Let me share my own findings with you. I visited the engine-room (housing the generator set) and walked about it with a dosimeter. Gamma-radiation in different

parts of the building was within 500–800 mr an hour, or 1 per cent or less of the dose rate after the accident. The area of the control console was not damaged (the level of gamma radiation is 4 mr an hour). I asked where operator Khodemchuk had died during the accident. (He was reported to have been buried under the debris; his body was never found.) It turned out that the shift supervisor had sent him to the master circulation pump department. Why? The answer is not clear even today.

Inside the sarcophagus I saw numerous holes drilled in the wall situated a mere 6m from the active zone of the reactor. Frankly, I found it hard to believe that people could get that close to the radioactive volcano. My dosimeter showed 100 mr an hour. Drillers work in 3 or 4 shifts. One metre a shift is considered to be good progress, but sometimes no more than several centimetres are drilled. The daily personnel body-dose there is 0.3 rem (roentgen equivalent, man).

The building of the crippled generating unit is huge and its several compartments are linked in a maze. The roughly 400,000 m^3 of concrete poured into the damaged building during the construction of the Sarcophagus has created extra obstacles for drillers working there today. Sometimes you can walk (and sometimes you have to run) along narrow corridors. Their walls are covered with thick lead panels and the floor is covered with gauze saturated with decontaminating fluids.

Then, suddenly, you see a warning sign: "Stop! 200 roentgen an hour!" blocking the entrance to a passage or stairwell. At such moments you become aware of the presence of radiation. You understand how difficult it is to control it and realize how indebted we are to the brave men working there, day-to-day, until they receive the maximum permissible dose of radiation. Kambulov told me that the personnel working inside the sarcophagus had been replaced five times during 1988.

Research at and around the Chernobyl nuclear power plant continues. A group of radiology experts from the USSR Academy of Sciences is joined in the effort. Under the Norka (Mink) project, a first batch of these furry animals will be released into the plant's coolant pond, which is rich in fish. The fish-breeding farm there is to resume operation.

Changes in the flora and fauna of the zone are of special interest to researchers. For the time being, a handful of enthusiasts are working on the greenhouse farm of the city of Pripyat, also a very interesting subject; they have many plans and ideas, but for the time being lack the necessary funds and instruments. But I believe they have started something. Chernobyl is a dark episode in the history of mankind which may yet serve as a world laboratory and so help prevent similar accidents. □

Understanding hydrogen bonds?

New measurements of simple complexes of hydrogen fluoride and nitrous oxide have thrown light on the properties of two quite distinct kinds of hydrogen bonds.

ALL of us know that hydrogen bonds are important, for example in making the structure of water peculiar, in holding the two strands of DNA together or in accounting for the ways in which protein molecules cohere with themselves, but few of us know what hydrogen bonds are like. That is why we should all be grateful to Christopher M. Lovejoy and David J. Nesbitt of the US National Bureau of Standards at Boulder, Colorado, for another set of infrared (or vibrational and rotational) spectra of unlikely molecular complexes — this time between nitrous oxide (N_2O) and hydrogen fluoride (HF), as reported in the latest issue of *J. Chem. Phys.* (90, 4671; 1989).

Bonding, very much a chemist's concept, suggests interatomic links that endure for the rest of time. Tie atoms of carbon and hydrogen together and you have an interatomic link that may at one stage be part of a benzene ring and, at another, a constituent of some methane molecule discharged from an oil-well in the Persian Gulf. Bonds like that, of course, are distinguished by the energy required to break them, essentially of the order of one electron volt (1 eV), which is equivalent to a Boltzmann temperature of 10,000 K in round numbers. Hydrogen bonds, by contrast, are less durable by a factor of 100, which means that they are breaking and reforming repeatedly at room temperature. But none of that implies that hydrogen bonds are not bonds: it is partly that they are labile, partly that they are in danger of being confused with other kinds of inter-molecular interactions, van der Waals forces for example.

The distinction, admittedly, is not nearly as sharp as one might wish. Take, for example, a mixture of argon and hydrogen chloride (Ar and HCl). Pure argon is a near-perfect perfect gas, but even quite small proportions of HCl will make the gas laws go awry — the virial coefficients that measure departures from Boyle's law (PV is a constant) are surprisingly large and often opposite in sign from what might be expected. Happenings like that should have pointed to the likelihood that atoms of the inert gases can form compounds long before they were discovered.

The natural link between HCl and Ar atoms is that in which the H is situated geometrically between the two heavier atoms, the Ar and the Cl. There, as a

simple proton, it can alternatively rob either the rare gas atom or the chlorine ion of an electron (reverting to atomic status itself). That is how Pauling would have put it in the first edition of *The Nature of the Chemical Bond*. What the experiments show is something different. Ar-HCl is relatively stable, but there is also evidence that Ar-ClH has a transitory existence. Plainly, what matters most is the polarizability of completed electron shells, with the complication (for those who solve Schrödinger wave equations for a living) that it is not easy to know what to make of the Born-Oppenheimer approximation (which supposes the motions of all nuclei to be slow) when there is a rogue proton to account for.

That is why it seems safer to think of hydrogen bonds as dynamic structures, which is nicely illustrated by the tendency of molecules such as hydrogen fluoride (HF) to form dimer molecules. Why dimers? And, if so, how is it determined which of two HF molecules contributes the intermediate proton to the complex? The predictable answer, of course, is neither. At any reasonable temperature, one or other or both molecules will be rotating. For practical purposes, the F atoms will be the dogs and the H atoms their tails. If interposed protons make for binding in some degree, a viable $(\text{HF})_2$ complex will be a four-atom molecule in which one or other of the hydrogens will be roughly half-way between the two fluorines for much of the time. A little thought, of course, will show that there are two ways in which that may be accomplished — the HF molecules may be spinning in the same or in opposite directions. That means that there are two states for the hydrogen bonded dimer, whose energy differs by a frequency measured in MHz. There was an elegant paper in *J. Chem. Phys.* a few months ago that demonstrated just that.

What Lovejoy and Nesbitt have to say has much in common with that tale, but relates to the dimers formed by hydrogen bonding between HF and N_2O (nitrous oxide) — not the symmetrical molecule that might be imagined, but one that has a nitrogen atom in the middle.

There are two ways in which hydrogen bonds may be formed: the H of HF is adjacent either to the terminal oxygen or to the terminal nitrogen. It turns out that the link with the terminal oxygen is the more stable, but that the resulting five-

atom hydrogen molecule is bent. In the language of the elementary chemistry text-books, the terminal oxygen has two 'lone-pairs' of electrons, sticking out sideways from the linear N_2O molecule at an angle not very different from the tetrahedral angle, with each of which an HF molecule may make an advantageous linear connection.

Lovejoy and Nesbitt have found spectroscopic evidence to bear out the existence of the other form in which the H of HF is bonded to the terminal N of N_2O . Their experimental technique is horrendous as, these days, these matters are. Hydrogen-bonded molecules are formed as a supersonic stream from a high-pressure nozzle (which has the virtue of cooling the the molecules almost to absolute zero) and their vibrational spectra recorded with a laser system with a resolution of roughly 6 MHz. The familiar problem of the analysis is to fit the lines in the rotational and vibrational spectrum of the possible molecules to those actually observed. Among the complications of the analysis are the need to take account of the distortions of both kinds of molecules under the influence of centrifugal forces, which stretch the linear complexes and straighten those in bent form.

The bearing of all this on hydrogen bonds in general is not as remote as it might seem. The benefits of a spectral analysis of the kind described by Lovejoy and Nesbitt is that it is possible to estimate the force constants of the bending motions in which the directions of the linear and bent hydrogen bonds are displaced from their equilibrium positions. What that analysis shows is that, while the linear hydrogen bond (FH-NNO) is the more stable, it is also the more rigid. All that is explicable in terms of simple arm-waving about the directionality of different kinds of hybridization between *s* and *p* orbitals.

All of this is, of course, a long way from the proper understanding of how strands of DNA are held together, let alone from an understanding of the structure of liquid water. But it is a lot better than nothing that it now seems possible to measure the mechanical properties of the most shadowy, if also the most important, of all chemical bonds. Nobody would suggest that the time has come to start calculating with hydrogen bonds as if they were familiar aliphatic CH bonds, but the time when that is possible may not be far off.

John Maddox

Shaping up for selective catalysis

John Dwyer

SELECTIVITY by molecular shape in the synthesis of organic molecules is obviously desirable, but how does it happen? On page 200 of this issue, Anderson and Klinowski¹ describe high-resolution NMR experiments showing for the first time the operation of a shape-selective process from inside a zeolitic microuniverse. These, and other shape-selective effects, are now widely exploited in petrochemicals and are being developed for wider use in chemical synthesis.

Zeolites are crystalline aluminosilicates consisting of tetrahedral AlO_4 or SiO_4 units linked through oxygens to generate open frameworks prescribing systems of pores and cavities with molecular dimensions. Access to the intracrystalline pore systems, which may be one-, two- or three-dimensional, is controlled by windows of peripheral oxygens. Most zeolites now in use as catalysts have windows with 8,

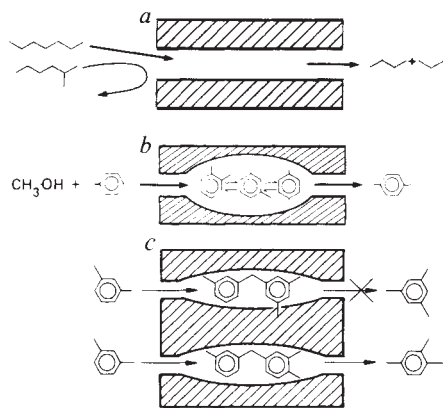


FIG. 1 Shape selectivity in catalysis arises from reactant (a), product (b) or transition-state (c) stereo-restrictions. (From ref. 5.)

10 or 12 oxygens in the constraining rings, and are classified as small (4–5 Å), medium (5–6 Å) and large-pore (7–8 Å) zeolites, respectively (Fig. 1). Even larger pores (18-rings) are available in the related AlPO_4 materials.

The sites active in acid-catalysed reactions arise from the presence of a negative charge on each AlO_4 unit of the zeolite framework, which must be compensated for by cations. When protic hydrogens serve that purpose, bridged hydroxyls, $\equiv\text{Al}(\text{OH})\text{—Si}\equiv$, with strong Brønsted acidity are produced, their activity depending largely on local composition and structure. Active sites may also be generated by the incorporation of multivalent cations or of occlusions of reactive metals, metal oxides or metal complexes.

Molecular shape-selective catalysis^{2–7} comes about because most of the active sites are in the intracrystalline pore regions, where there may be severe spatial

restrictions on reaction intermediates and the transport of molecules. Diffusion in micropores, referred to as configurational diffusion, is sensitively dependent on the sizes and configurations of both sorbed molecules and pore systems. The basis of controlled shape selectivity is the deliberate matching of the geometry and tortuosity of zeolite pore systems by the size and configuration of the reactant molecules, the product molecules or even the reaction intermediates.

Consequently there is shape-selectivity whenever access of a reactant to active intra-crystalline sites is inhibited. Thus over the small pore (8-ring) zeolite, CaA, *n*-butanol but not *isobutanol* is dehydrated because *isobutanol* cannot readily penetrate the 8-ring. Over the large pore X zeolite (12-ring), both isomers are dehydrated. Similarly, normal, but not branched, hydrocarbons may be cracked in erionite (8-ring) (Fig. 1a).

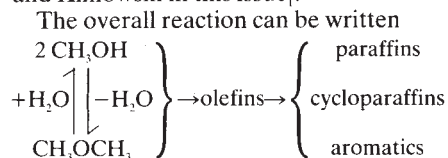
Particular product molecules will be selected over others when they diffuse rapidly through the crystal pores and exit into the fluid stream. The delayed molecules may then be converted to less bulky molecules by further reaction. For example, in the methylation of toluene over H-ZSM-5, the yields of *p*-xylene can be enhanced because its molecules diffuse more rapidly than the other isomers.

Shape selection may work on reaction intermediates when spatial constraints in the environment of reactive sites prevent the formation of a bulky molecular transition state. Thus the bimolecular disproportionation of *o*-xylene proceeds through a relatively large diaryl intermediate, whereas monomolecular isomerization to *m*- and *p*-xylenes requires consecutive 1–2 methyl shifts with a smaller transition state. So, as the size of the zeolite cavity is reduced, the rate of disproportionation of xylene relative to that of isomerization is considerably reduced. Quantitative treatments of shape selectivity based on reaction/diffusion analysis are available⁸.

This analysis has been used to calculate the secondary product distribution, at low conversion, for three different initial xylene compositions from the reaction of toluene and methanol (Fig. 2). *Para*-xylene selectivity at low conversion is seen to increase with the value of the Thiele modulus, $\phi = l\sqrt{k/d}$ where l is the crystal size, k reactivity and d molecular diffusivity, but at higher conversion, *p*-xylene selectivity depends on both the Thiele modulus and on the relative rates of alkylation and isomerization. The influence of diffusion times on *p*-xylene selectivity is demonstrated by experiments in

which diffusion times are increased by changing crystal sizes, by chemical modifications and by 'coke' deposition⁹.

Shape-selective catalysis is widely exploited commercially. The objective of several processes is aromatization, the most widely studied of which is the direct conversion of methanol to gasoline (MTG), which provides a route to gasoline from coal and natural gas, and which is the subject of the paper by Anderson and Klinowski in this issue.



The second stage of this reaction can be explained by carbenium-ion chemistry in which olefins oligomerize and cyclize to cycloparaffins, which are then converted to aromatics after hydrogen transfer. With medium-pore (ZSM-5) zeolite, there is a

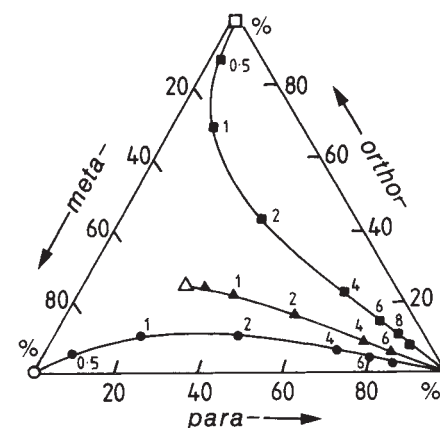


FIG. 2 Stereo effects in the catalysis zeolite H-ZSM-5 of the methylation of toluene: $\text{C}_6\text{H}_5(\text{CH}_3) + \text{CH}_3\text{OH} \rightarrow o-, m- \text{ or } p\text{-C}_6\text{H}_4(\text{CH}_3)_2 + \text{H}_2\text{O}$. As the Thiele modulus (see text), is increased from 0.5 to 8, so is the preference for the *para* isomer. (From ref. 8.)

cut-off between C_{10} and C_{11} with a wide range of feedstocks. These results are interpreted by assuming that a similar pool of olefins is generated from all feedstocks and that dehydrocyclization to aromatics takes place in the channel intersections of ZSM-5 which provide the largest free space (about 9 Å diameter), but can accommodate transition states with more than 10 carbon atoms only with great difficulty. The results of Anderson and Klinowski are in general agreement with these views, but demonstrate that C_{12} species can be generated in the crystals but cannot exit.

A comparison of the C_6 and C_{10} isomers in the solid and gas phases also provides direct evidence for product selectivity, but the suggestion that a further kind of shape selectivity is required to explain the distribution of C_{10} isomers requires further work: it should be established first that there are no significant differences in the

sizes of transition states or in chemically determined kinetic paths. Although configurational site preferences have been suggested⁹ as factors in highly selective zeolite catalysis, shape-selectivity may not necessarily be involved, but merely selection factors known in other catalysts.

The first step in the MTG process is still the subject of discussion¹⁰. Several have supposed that surface methoxy species are involved; it is interesting that Anderson and Klinowski find no evidence for these in their NMR studies. This may be a function of timescale, but obviously requires clarification, as does the significance of carbon monoxide, which is seen clearly in these NMR studies.

Several other effective uses of shape-selective catalysis are in operation or development. For example, small paraffins can be converted directly into aromatics (usually) by dual-function medium-pore zeolites. An example is the Cyclar process which uses ZSM-5 modified with gallium oxide to convert C₃ and C₄ alkanes into aromatics. Further improvements can be made by addition of oxygen. Very high selectivities in the conversion of hexane into benzene are possible using a platinum-loaded zeolite L (Aromax process). It is proposed that these high selectivities involve pore organization of *n*-hexane arising from confinement effects in the zeolite pores⁹.

The upgrading of gasoline, which becomes increasingly important for lead-free petrol, can be achieved by including medium-pore zeolites into the fluid-catalytic-cracking cycle. Selective cracking of normal hydrocarbons in the presence of branched hydrocarbons, for example, using reactant shape selectivity is used both to increase octane in smaller hydrocarbons and to improve the properties of fuel and lubricating oils.

Current aromatics processing also uses shape selectivity extensively. The medium-pore zeolite, ZSM-5, is widely used to process C₈ streams with minimum loss of xylenes by disproportionation. Similarly, ZSM-5 chemically modified to obtain an optimal balance in the rates of methylation of toluene and of the diffusivity of the product xylene is used in the selective synthesis of *p*-xylene from methanol and toluene. Ethyl benzene and *p*-methyl ethylbenzene are produced

almost quantitatively by ethylation of benzene or toluene respectively with ethene. Both processes avoid the problem with Friedel-Crafts acids and make use of shape selectivity to avoid further alkylation or transalkylation (by restriction of the transition states). Selective *p*-methyl ethylbenzene production depends on product selectivity.

The future for shape-selective microporous catalysis looks very promising. New materials are constantly being synthesized and interest is spreading to a wider range of chemical processing. For

example, shape-selective reactions involving phenols, amines, chlorobenzenes, heterocyclics, Beckman and Fries reactions, peroxide chemistry and the like have been widely reported. In the hydrocarbon field, the direct conversion of methane must surely be close. Moreover, the discovery of the first zeolite, zeolite β , with chiral structure may offer exciting prospects in organic synthesis. □

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MEMORY

Must what goes up come down?

Richard G.M. Morris and David J. Willshaw

THE idea that changes in the efficacy of synapses are the way the brain stores information has been the mainspring for a great deal of work on synaptic potentiation, particularly the physiological phenomenon of long-term potentiation (LTP) in the hippocampus. On page 215 of this issue¹, Stanton and Sejnowski offer provocative new evidence that synaptic efficacy may also obey the time-honoured principle that what goes up must also come down. Using slices of hippocampal tissue maintained *in vitro*, they show that particular patterns of neural activity can give rise to an associative long-term depression of synaptic efficacy. The existence of such a capacity in the hippocampus has long been suspected, and indeed is required by certain neural-network models of associative memory² and synaptic connectivity^{3,4}. It is already known to occur in the cerebellum⁵.

The phenomenon of LTP is now widely agreed to be rapidly induced, fairly long lasting, synapse-specific and associative. These properties, particularly those of synapse specificity and associativity — whereby changes are induced only when presynaptic activity and postsynaptic depolarization occur at the same synaptic terminals^{6,7} — all suggest that LTP is a good neurobiological model of memory⁸. But a problem with synapses that can only increase in efficacy is that the range of associative relationships which the memory system can encode is limited, an example being whether memory can be updated when the relationship between two events changes. A second problem is that memory systems of this type can reliably store only a certain amount of information, and one question is whether the facility to decrease as well as increase synaptic strengths can improve performance. A third point is that LTP does not last indefinitely^{9,10} and is sometimes induced at activated terminals together with a depression at synapses where presynaptic activity has been absent^{11–13}. The

discovery of associative synaptic depression is relevant to all these points.

In their paper in this issue¹, Stanton and Sejnowski explore the effects of varying the temporal relationship between 'conditioning' bursts and 'test' stimuli in the CA1 region of the hippocampus. The bursts consisted of 5 pulses at 100 hertz spaced at 200-millisecond intervals, and the test stimuli were single pulses. The authors observe LTP when these two inputs are in phase, and measured it using low-frequency test stimulation for periods of up to 3 hours. But if the same inputs are delivered out of phase such that the single test pulses occur exactly 100 milliseconds after one burst and 100 milliseconds before the next, the test pathway shows a long-term depression (LTD). This change is particularly noticeable in the population spike (an extracellular measure of the number of cells firing) but only smaller changes occur in the initial slope of the field potential. Because the latter is generally held to be a better extracellular measure of synaptic efficacy, this difference raises the possibility that the observed LTD is restricted to the coupling between current input to the soma and the spike-generating mechanism rather than a true localized decrease in synaptic weight. (An analogous form of LTP known as e.p.s.p.-spike potentiation has also been described⁸.)

Stanton and Sejnowski explicitly make the point that they "searched for conditions under which the stimulation of a hippocampal pathway rather than its inactivity" could produce LTD. This is, of course, crucial because presynaptic stimulation is an ideal way of achieving a synapse-specific change. It is not clear to us, however, whether Stanton and Sejnowski have fully explored the effects of omitting the single test pulses in the out-of-phase conditioning regime. This would restrict the inducing stimulation to the conditioning pathway and might fulfill the conditions for producing heterosynaptic

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depression — depression at inactivated synapses. Stanton and Sejnowski state that such stimulation “did not significantly alter the amplitude of responses to the test input”, but present no relevant data. In their absence, the claim that these data show an associative rather than a heterosynaptic depression must remain open to question.

These problems are relevant to an exciting second series of experiments in which Stanton and Sejnowski¹ consider a possible neurophysiological mechanism of LTD. Test stimuli given 100 milliseconds after conditioning bursts will necessarily arrive when the postsynaptic neuron is hyperpolarized. To explore whether this conjunction is crucial for eliciting LTD, they used intracellular techniques to compare the effects of pairing afferent activity and hyperpolarizing current injections with the effects of hyperpolarization alone. Their results indicate that presynaptic activity is necessary for producing LTD; that is, that this type of synaptic depression is associative.

Interestingly, similar conditions have been explored previously by Malinow and Miller¹⁴, who found that postsynaptic hyperpolarization blocks LTP. In that study, however, higher-frequency test stimulation was used in conjunction with the hyperpolarization. This may have raised the intracellular dendritic voltage into a kind of no-man's land — above the level at which LTD may occur but below the level sufficient for inducing LTP. Malinow and Miller, however, also used a 1-nanoamp hyperpolarizing current throughout the monitoring phase of their experiment to increase the amplitude (and thus ease of measurement) of their evoked potentials. With the wisdom of hindsight, we can now speculate that this unintended conjunction of hyperpolarization and low-frequency test stimuli might have been the cause of responses in their control group showing the gradual decline in amplitude with time which they found.

How does the discovery of associative LTD bear on models of associative memory? It is clear that the rule determining how synapses change in response to particular conjunctions of pre- and postsynaptic activity is an important determinant of memory performance. Models in which negative changes are also allowed perform better than a strict hebbian model, in which synaptic changes are restricted to increases, although an absolute limitation in performance still remains. A second point concerns the conditions under which associations are formed and the capacity of associative memories to represent changes in the relationship between events. Consider the situation in which one stimulus (such as a tone) has long been associated with a reinforcing stimulus (such as food) but is now presented alone. The effect of such

training is for the memory of food evoked by the tone to decline in proportion to the number of nonreinforced tone presentations. A strictly hebbian mechanism cannot explain this change. The capacity for synapse-specific associative depression, however, provides one simple explanation for the change in memory representation (although not the only one: see ref. 15), given that it is the presentation of the tone — the presence of presynaptic activity rather than inactivity — which actively reduces its capacity to evoke the memory of food.

A further implication of the discovery of associative LTD concerns the time-course of LTP. Long-term experiments in which the time-course of LTP has been studied have typically found a steady return to baseline over time^{9,10}. If the conjunction of presynaptic stimuli with postsynaptic hyperpolarization causes synaptic depression, it is not unreasonable to imagine that this would happen frequently during normal neural activity. Chronic studies of LTP, therefore, may be plagued by the problem that the conditions for producing LTD are inadvertently but unavoidably produced by the normal activity of the hippocampus. Thus, the true picture about the maintenance of LTP may be that the synaptic changes are nondecremental unless the specific conditions for actively reducing synaptic efficacy are realized. Studies of the time-course of both LTP and now LTD must in future be conducted under conditions ensuring that measurements of the time-

ANTHROPOLOGY

A tale of three cities

J.S. Jones

THE populations of Hiroshima and Nagasaki are, for good historical reasons, genetically the best known in the world. A new insight into the genetics of these cities has been gained from the inhabitants of a third, as yet imaginary city: a sort of mendelian Laputa somewhere in South America which, like its swiftian counterpart, is an allegory of the societies which will emerge as the people of the developing countries rush into new conurbations. Only a few months after the atom bombs were dropped, biologists began to take advantage of this unnatural experiment in human genetics, and since 1948 the children of the survivors of Hiroshima and Nagasaki have been subjected to increasingly sophisticated tests for new mutations. The first final reports on this programme are now beginning to appear.

Early fears of a leap in the mutation rate were unfounded and it seems that man-made radiation is always likely to be less important in mutagenesis than is that from natural sources^{1,2}. However, so much

information has been gathered that the genetic history of the two cities can be inferred in some detail. The genes of their modern inhabitants still echo the complex mating patterns of their distant ancestors, a finding which should instil a certain gloom into those who hope to use human molecular diversity to test the predictions of population genetics theory.

Children born in the devastated cities were divided into two groups: those in which both parents had been within 2 km of the bombs, and a control group in which one or both parents were further from the explosions. A study of more than 70,000 pregnancies gives no evidence that irradiation increases birth defects³. The survival to adulthood of the children of irradiated parents does not differ from that of controls, and there is no significant increase in the incidence of chromosomal abnormalities⁴. A survey of 667,404 proteins detected by electrophoresis among the exposed group uncovered three new mutations. In the controls, the same

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number of mutations was found in 466,881 tests⁵. Mutation is such a rare event that, despite the huge numbers of tests, statistical errors mean that a doubling of the mutation rate in the exposed population cannot be excluded. For this reason, and because estimates of the radiation intensity of the Hiroshima bomb have recently been revised, the United Nations estimate of the amount of radiation needed to double the human mutation rate is still based mainly on research on mice and, at 100 rads per lifetime, is four times more cautious than that proposed by those working in the bombed cities^{6,7}. Humans may be substantially less sensitive to radiation than projected from data using mice⁸, and effects of radiation in producing cancer are of much more immediate importance than are its possible long-term effects on the germ line⁷.

About a million genes have now been counted in Hiroshima and Nagasaki. As well as its importance to studies of mutation, this mass of information has produced an insight into genetic history. Nearly all the 30 enzyme loci used have rare alleles, each present at a frequency of around one in a thousand⁹. The two cities have about 120 rare alleles each. The neutral theory of molecular evolution (which ascribes genetic diversity to a balance between the random origin and loss of alleles with no effects on fitness) can, in principle, predict the distribution of such alleles⁹. Any deviation from its predictions might indicate the action of natural selection. At first sight, the numbers of rare alleles in the two cities are higher and their patterns of frequency distribution very different from those expected. The neutral theory, however, depends on the assumption that the population under investigation is a homogenous one. The data from Hiroshima and Nagasaki show that this assumption is unjustified for modern humans.

The first cities appeared only about 5,000 years ago¹⁰, and urban epidemics meant that until the eighteenth century they could not sustain themselves without continued immigration from the countryside. The genetic implications of this history can be tested by looking at the population structure of societies still living in a pre-urban age and asking what kinds of cities will emerge when they finally join the modern world. In South America, each Indian tribe is genetically isolated from its neighbours by geographical and social barriers. These barriers are impermeable enough to allow individual tribes to accumulate unique genetic variants or 'private polymorphisms' which do not spread to adjacent groups. Many of these are as characteristic of a particular tribe as are surnames in Europe. Within a tribe

the private polymorphisms may reach appreciable frequencies, although among the aboriginal population of South America as a whole each variant is extremely rare.

This pattern is the key to understanding the modern populations of Hiroshima, Nagasaki and other cities. In a computer-generated artificial city¹¹ made up of migrants from different South American Indian tribes, the distribution of gene frequencies is remarkably close to that found in urban Japan. The population of this mythical town has far more rare alleles than expected on the neutral theory; but this does not, as might naively be argued, reflect an increased mutation rate or the action of natural selection.

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Makiritary Indian children — founders of tomorrow's cities?

Instead it is a remnant of a history of private polymorphisms within single tribes each of which, when agglomerated, becomes extremely rare. The genetic structure of the modern city reflects its tribal past.

Japan was first settled about 30,000 years ago. By 8000 BC the Jomon culture was widespread, but this was replaced in parts of Japan at about the time of Christ by the rice-growing Yayoi people. Nagasaki is near the southern tip of Japan, where the Jomon culture persisted for longer than around present-day Hiroshima. Ancient writings show that for much of its history Japan was divided into a series of warring clans not unlike those found in South America today. The large number of rare alleles within each of the modern cities almost certainly reflects the agglomeration of ancient and genetically divergent tribal groups.

The differences in the identity of these alleles between Hiroshima and Nagasaki

are a remnant of prehistoric conflicts and barriers to mating among at least five (and perhaps many more) ancestral clans. Although Nagasaki was one of the few ports open to the outside world during Japan's long self-imposed isolation, it had no more of an influx of foreign genes during historical times than did Hiroshima. The voices of remote ancestors echo more loudly through these two cities than do the voices of more recent invaders.

Ancient genetic history is also preserved in the cities of modern Europe, although its record has not been as fully unearthed as has that of Japan. Italy has been divided into genetically distinct zones on the basis of blood groups and enzymes¹². These concur to a great extent with the history of foundation of its cities. The modern Sicilian town of Messina is the successor of one of the centres of Magna Graecia, the empire set up after 800 BC as a result of the explosion of culture and population on the Greek mainland. The genes of its present inhabitants still show strong affinities with those of modern Greece. Further north in Italy, the Etruscans built cities of up to 400,000 people long before the time of Christ, and the genes of this extinct culture are still manifest as a biologically distinct region around Orvieto. There are traces of the speech of these ancient peoples in the dialects of modern Italy. Some other linguistically distinct Europeans — such as the Basques — also differ genetically from surrounding peoples¹³. Just as in Japan, this may reflect genetic changes during a period of tribal isolation thousands of years ago. We are beginning to understand the language of the

genes, and their message may tell us as much about our history as do the spoken and written fragments of ancient dialects. □

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Anatomy of the magma reservoir

Ken C. Macdonald

FULLY two-thirds of the Earth's crust is created by spreading at the mid-ocean ridge, a feature likened by the late Bruce Heezen to "a wound that never heals". Most of the molten rock that solidifies into oceanic crust resides within a shallow magma chamber beneath the axis of the

floor by Detrick *et al.*². Yet this pool of magma cannot be resolved in the tomographic images, which means that it is probably not more than a few hundred metres thick. Thus, the axial magma chamber may resemble a mushroom in cross-section with a narrow stalk of partial

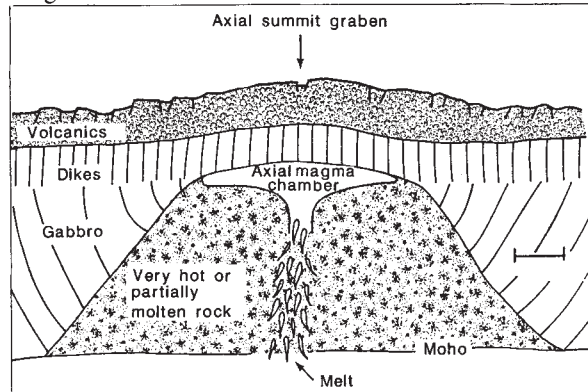


FIG. 1 Schematic cross-section of the East Pacific Rise based on seismic and gravity results (modified from refs 1–5). A halo of hot rock with at most a few per cent partial melt surrounds a much smaller mushroom-shaped magma chamber (more than 50 per cent melt) and buoyantly supports the overlying ridge. Moho: the Moho discontinuity at the base of the crust.

ridge before erupting onto the sea floor or freezing onto the edges of the parting plates. For the first time, as reported by Burnett, Caress and Orcutt on page 206 of this issue¹, this site of sea-floor creation has been imaged using sophisticated acoustic tomographic techniques on the East Pacific Rise near 13° N.

Using over 2,500 source–receiver pairs, the authors have determined the shape and physical properties of the axial magma chamber in much the same way that clinicians use computer-assisted tomography (CAT) to examine the interior of the human body. One disadvantage is that the sources and receivers do not surround the axial magma chamber on all sides as they do in medical CAT scans, but are confined to the sea floor and the overlying water. Nevertheless, the images of Burnett *et al.*¹, in conjunction with other seismic^{2–4} and gravitational⁵ measurements, provide the best glimpse yet of the axial magma reservoir.

Taken together, these studies show that the mostly molten chamber (containing more than 50 per cent of melt) is actually quite small; but it is surrounded by a wider reservoir of very hot rock with at most a few per cent of partial melt, which reduces the sound velocity by 0.5–1 km s⁻¹ relative to surrounding cooler sea floor (Fig. 1). This halo of very hot (over 1,000 °C) or slightly molten rock is at least 6 km wide¹ and isostatically supports a ridge about 200–400 m high and 8 km wide along the axis of the East Pacific Rise⁵. A thin pool of nearly 100 per cent melt approximately 4 km wide caps the top of this broader reservoir of hot rock^{2,3} and provides the large acoustic impedance contrast needed to explain the very bright reflector observed 1.4 km beneath the sea

melt feeding a thin lens of pure melt 4 km wide (Fig. 1)^{1–4}.

The axial magma chamber, however, may not be stable in time or continuous along the strike of the East Pacific Rise^{2,6}. Shaded-relief images of the sea floor where the tomographic work was completed indicate a northward propagation of the feature at a rate of approximately 500 mm yr⁻¹ that has resulted in a shift of

the ridge axis to a location 1–2 km eastward in the past 100,000 yr (Fig. 2). This has resulted in a short ridge segment bounded by two small offset (by less than 2 km) overlapping spreading centres near 12°37' N and 12°54' N (ref. 7). Remarkably, these tiny, short-lived discontinuities in the structure of the ridge mark small but clear gaps in the axial magma chamber², although the broader reservoir of hot rock simply bends to follow the ridge¹. Along-strike variations in the axial magma chamber are manifested in subtle changes in the structure and morphology of the ridge: where the chamber disappears, so does the axial summit graben (a narrow cleft which marks the axis of the ridge) and the cross-sectional shape of the ridge becomes narrower and steeper⁸. On a 20–100 km scale, the depth of the ridge axis undulates up and down like a serpent, the low points occurring at gaps in the axial magma chamber, which coincide with the location of ridge-axis discontinuities such as overlapping spreading centres⁷. These discontinuities thus reflect a fundamental segmentation of the mid-ocean ridge^{7,9–11}.

The story becomes more complex when rock samples collected along the ridge are analysed. Langmuir *et al.*⁹ find geochemical evidence for segmentation on an even finer scale along the ridge (10–50 km) at structures termed devals, whose tectonic signatures are very subtle. They propose that all discontinuities, even down to the scale represented by devals, represent boundaries between regions or ridge seg-

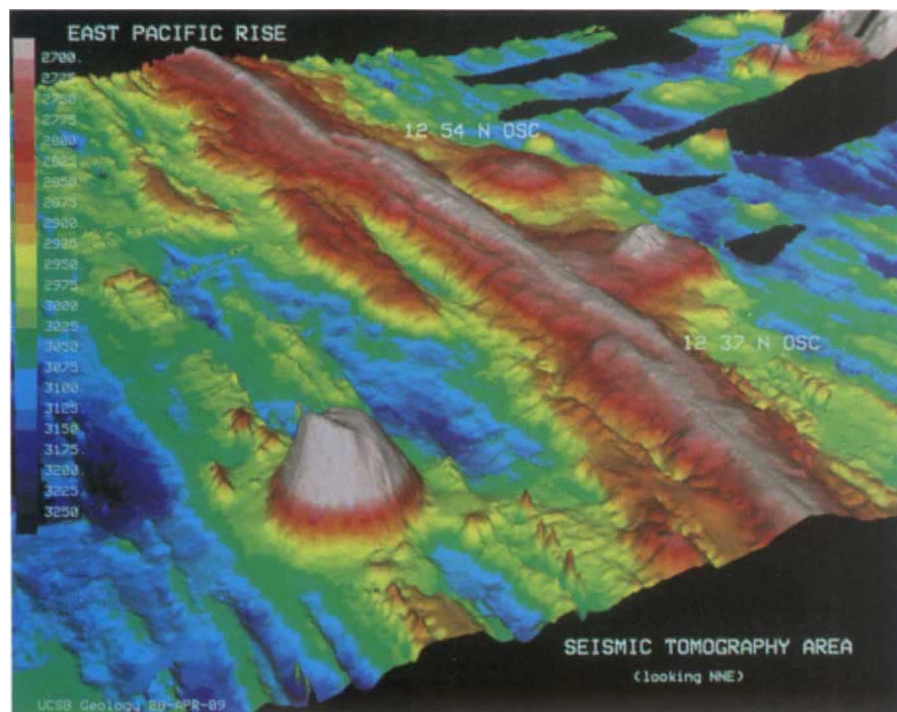


FIG. 2 Colour shaded-relief image of the East Pacific in the tomography area using combined SeaMARC II and Sea Beam bathymetric data (with vertical exaggeration of 6, produced by S. P. Miller using NECOR software and unpublished data of K. C. M. and P. J. Fox). The ridge segment is bounded by small offset (<2 km) overlapping spreading centres at 12°54' N and 12°37' N, as shown. The axial magma chamber is present beneath most of the Rise including a deva at 12°46' N, but deepens and finally disappears at the two overlapping spreading centres.

ments with separate magma supplies⁹. But multi-channel results indicate that the reflector interpreted to be the roof of the axial magma chamber is continuous beneath most devals², so that fine-scale geochemical segmentation seems to conflict with seismic data. The tomographic results may help to resolve this dilemma if the axial low-velocity zone is primarily hot rock with a low percentage of partial melt and the true magma chamber is only a thin lens fed by a narrow stalk of pure melt (Fig. 1). The scale over which magma can mix in a long chamber (20–100 km) of small cross-sectional dimension will be short, especially if the magma resides only briefly in the chamber before eruption⁷. Thus a single continuous chamber may keep distinct contributions of partial melt from the upper mantle separate until the time of eruption, and the smallest scale (10–50 km) segmentation of the ridge may be governed by a characteristic mixing length within the axial magma chamber. So when geochemists assert that even the shortest ridge segments represent regions

of separate magma supply⁹, and seismologists and tectonicists see evidence for a much more continuous reservoir along the strike of the ridge^{2,6,8}, they may all be correct. The results of another more recent tomographic experiment on the East Pacific Rise near 9°30' N, where the axial magma chamber is thought to be larger, may clarify these issues¹². □

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EPSTEIN–BARR VIRUS

Synthesis for a complex role

Bill Sugden

EPSTEIN–BARR VIRUS (EBV) a herpesvirus infecting most of humanity, is nevertheless rarely associated with cancers. It often causes infectious mononucleosis, but this lymphoproliferative disease is self-limiting. The discovery and properties of EBV were discussed in News and Views last month by John Galloway¹, and new approaches to understanding its role were discussed at a recent meeting*.

It emerged at the meeting that our understanding of the role of the virus in human carcinogenesis has reached a new synthesis, derived on the one hand from the study of B lymphocytes immortalized by infection with the virus *in vitro*, and on the other by analysis of biopsies of EBV-associated Burkitt's lymphomas. B cells immortalized by infection with the virus *in vitro* consistently express at least eight viral proteins, six in the nucleus and two in the plasma membrane². Genetic evidence demonstrates that at least one of the nuclear proteins is required for immortalization of the B cell, and much circumstantial evidence indicates that at least two of the others are also required.

The thesis that multiple genes of EBV mediate immortalization of human B lymphocytes is countered by the observation that some, perhaps all, biopsies of Burkitt's lymphomas initially express on explantation only one viral protein³. This viral product, termed EBNA-1 (Epstein–Barr nuclear antigen-1), at least in cells

immortalized *in vitro*, participates in the maintenance and expression of viral DNA in the proliferating cell. The initial expression of only EBNA-1 in human tumour cells that can be propagated indefinitely has led to the antithesis that in Burkitt's lymphoma multiple viral proteins are not required in carcinogenesis and that EBV may be only a passenger in the tumour cell.

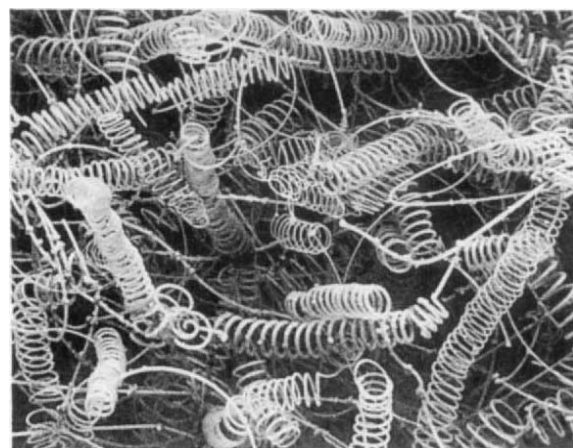
A resolution of these conflicting views has been detailed by George Klein

(Karolinska Institute, Stockholm), who proposes that the Burkitt's lymphoma cell is a long-lived resting B cell whose quiescence is interrupted as a result of its activation of the oncogene *c-myc*. Most B cells infected by EBV are blasts (large, proliferating cells) that express lymphocyte-activation markers, eight or more viral proteins, and are targets for cytotoxic T cells. In Klein's synthesis, EBV infects a particular cell (possibly a pro-B cell, a precursor in which no immunoglobulin rearrangements have occurred) in which translocations can occur that rarely lead to the activation of *c-myc* by its juxtaposition to an immunoglobulin locus. The role of the virus in lymphoid carcinogenesis is to expand the pool of cells in which these chromosomal rearrangements can occur. It is necessary even for cells with an activated *c-myc* to convert to resting B cells to escape immune surveillance. The normal, resting B cell lacks lymphocyte-activation markers, expresses only EBNA-1 and, as a consequence, is not a target for cytotoxic T lymphocytes that recognize other viral gene products. The proliferative capacity of this resting B cell that is not a terminally differentiated cell would be limited by the normal immunological controls exerted on this cell type. Activation of *c-myc* would abrogate these normal controls and permit an unregulated expansion of this EBV-positive cell whose surface markers mimic those of a resting B cell.

This model has been synthesized from information gleaned from diverse areas of research on EBV. Burkitt's lymphoma is associated both with infection by the virus and with malaria (D. Burkitt, Gloucester; see ref. 1). It has been shown that during the acute phase of malaria there are five-fold more virus-immortalized precursors in the bloodstream than during conva-

Ceramic microcoils sprung for action

THESE are not rusting parts of defunct cars, but rather microscopic coiled fibres of Si₃N₄ grown by Seiji Motojima and colleagues of Gifu University, in Japan (*Appl. Phys. Lett.* **54**, 1001–1003; 1989). The fibres were grown by chemical vapour deposition on graphite painted with iron. The coiling was completely unexpected and did not occur with other substrate paints. Coiling seems to be best with fibres 0.5–1.0 µm in diameter, those less than 0.1 µm not coiling at all and thicker ones (3–5 µm across) coiling irregularly, with a narrow pitch or as a spiral. The coils are flexible, as their appearance suggests, being extendable to three times their normal length. They are readily detached from the substrate, stable at high temperatures and resistant to corrosion, so that many uses can be imagined: as flexible packing material in harsh environments or flexible microfilters, for example. (The figure is 135 µm across; courtesy of S. Motojima). □



* Epstein–Barr Virus: The First 25 Years Oxford, 9–13 April 1989.

lescence (K. Lam, Royal Postgraduate Medical School, London). This finding is consistent with the notion that EBV infection, together with malaria-associated T-cell immunosuppression, provides an exceptional reservoir of infected B cells in which a *c-myc* translocation could occur. The T-cell response affected by malaria is apparently directed at several of the viral genes expressed in cells immortalized *in vitro*. Cytotoxic T-cell clones that recognize B cells immortalized only by strain A of EBV, but not by strain B, have been shown to recognize two viral proteins (EBNA-2 and EBNA-3A) found in the nucleus. These findings come from experiments performed by introducing expressed viral genes from strain A that encode these nuclear proteins into B cells immortalized by strain B that are not recognized by the T-cell clones and finding that the recipient B cells subsequently are targets for killing (D. Moss, Queensland Institute of Medical Research). As long as these gene-transfer experiments do not affect the expression of relevant surface molecules of the major histocompatibility complex, they indicate that T-cell recognition of EBV-infected cells could be similar to the recognition of influenza-infected cells⁴. Biopsies of Burkitt's lymphomas often do not express EBNA-2 or EBNA-3A³ and thus could escape the T-cell recognition described by Moss.

The expression of EBNA-2 in cells has been found to increase the expression of another viral protein, latent membrane protein, which itself can induce the expression of several activation antigens (S.D. Abbot, University of Birmingham; E. Kieff, Harvard Medical School). This protein is often not found in biopsies of Burkitt's lymphomas³, in agreement with the failure of these lymphomas to express B-cell activation antigens.

A candidate for a cell in which *c-myc* translocation to an immunoglobulin locus can occur has been identified as an EBV-immortalized pro-B cell derived initially by infection of cells from fetal liver⁵. These cells yield different translocations involving the immunoglobulin H locus on propagation in culture (E. Altiok, Karolinska Institute). They also express B-cell activation antigens and at least seven EBV proteins. If they do represent the virus-infected precursor of a Burkitt's lymphoma, then development of this tumour will probably involve the loss of expression of activation antigens and some viral proteins during differentiation.

This proposed complex role for EBV in carcinogenesis will require further diverse tests before it serves as a thesis to be reconciled with new and differing views. Evidence for the association of the virus with B-cell lymphomas in transplant recipients and in people with AIDS has accumulated and indicates its direct role in carcinogenesis in severely immuno-

compromised hosts (D.H. Crawford, Imperial Cancer Research Fund, London; G. Lenoir, IARC, Lyon). By contrast with Burkitt's lymphoma cells, these tumour cells express both activation markers and at least eight viral proteins. This direct role of EBV lends support to the notion that it contributes causally to Burkitt's lymphoma. Its role in Burkitt's lymphoma may eventually be substantiated by testing in people a vaccine that has been found to protect New World monkeys

from developing lymphomas following intravenous administration of EBV (M.A. Epstein, University of Oxford). □

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No new fusion under the Sun

ELECTROCHEMICALLY induced fusion^{1,2}, now a subject of popular debate, is not a new notion. In the 27 April issue of *Nature*, extracts were printed in the News and Views section³ from the debate surrounding the claim during the 1920s of Peters and Paneth^{4,5} that ordinary hydrogen absorbed in palladium fuses to form helium. In his news story in the same issue⁶, Steven Dickman mentioned contemporary work of Tandberg at the Electrolux laboratories in Stockholm, which resembles closely the recent work, involving electro-chemical cells and the release of heat. My father, Torsten Wilner, worked with Tandberg in this and other nuclear research. I still have their notes which are a source of fascinating insights.

Tandberg's introduction to this work was indirect: he started in 1925 by trying to find metals suitable for sealing hydrogen in refrigerators. He became interested in palladium, but for its contrary properties — its permeability and large absorption capacity for hydrogen. He was struck by the work of Paneth and Peters^{4,5}, who were attempting to produce helium — for use in airships — by the "spontaneous nuclear catalysis" of hydrogen atoms in palladium metal, and who expected heat to be produced, although they did not pursue this aspect. As noted in Dickman's report⁶, the helium that they identified spectroscopically was in fact atmospheric, not the product of any nuclear reaction.

Tandberg followed up this work, using electrolysis to concentrate hydrogen at the palladium surface — effectively generating a high hydrogen pressure, he argued. The main benefit was to be the generation of energy: the original title of Tandberg's 1927 patent application for the process was "A method for the release of atomic energy", although this was later altered to "A method for producing helium", possibly as a concession to the patent office.

The patent was not granted, the main reason given being Tandberg's failure to answer the office's objections: what is "hydrogen of highest concentration"?; the mechanisms for the extraction of energy needed better explanation; and the description was not sufficiently complete for a competent person to use the invention.

After the discovery of deuterium in 1932, however, Tandberg and Wilner repeated the experiment, now using heavy water, probably supplied by Niels Bohr.

Their enthusiasm for fusion fired, the two continued with experiments using exploding wires, an approach now used in some laboratories to accelerate ions to sufficiently high energies to overcome Coulomb repulsion. High-voltage capacitors were discharged through thin palladium rods saturated electrolytically with deuterium. On the night of the first discharge, Tandberg asked my father to leave so that he would be able to report what had happened in the event of a nuclear explosion. Nothing spectacular did happen, although the noise of the discharges became a feature of the laboratory routine. No clear-cut results were obtained, possibly because good diagnostic techniques were not then available. I still have the notes and letters referring to the experiments, and some specimens of palladium rods and heavy water. All of these should be studied carefully.

Tandberg and Wilner continued their nuclear experiments in the basement of my father's home. They reproduced the famous experiments of Cockroft and Walton, artificially transforming ⁷Li into two α -particles by accelerated protons. In a subsequent experiment, bombarding a deuterium-saturated palladium sheet with accelerated deuterons⁷, they observed the fusion reaction of such interest now: $d + d \rightarrow {}^3\text{He} + n$. The product neutrons were slowed by paraffin wax and detected by the activation of silver. (Much of this work is discussed in a book written to celebrate Tandberg's versatility and achievements⁸.) Bertil Wilner

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QUANTUM OPTICS

Laser gain without inversion

Peter Knight

LASER action, the quantum amplification of light, is normally possible only if a population inversion is created between the two quantum states involved in the light emission. More excited atoms than unexcited atoms are necessary. Otherwise the atoms or molecules making the downward transition with the emission of a photon cannot overcome the reverse flow of atoms absorbing photons on the way up to the excited state: the losses exceed the gain and stimulated emission cannot grow. Most of the problems associated with constructing new lasers are connected with the need to pump sufficient densities of atoms into the upper level of a laser transition to create such an inversion. But Steve Harris now shows (*Phys. Rev. Lett.* **62**, 1033–1036; 1989) that it may well be possible to get net photon gain and laser amplification without the creation of a population inversion. To do this he exploits the effects of quantum interference in competing atomic transitions to suppress absorptive losses whilst leaving emission unaffected. Related ideas were proposed some years ago by V. G. Arkhipkin & Y. I. Heller (*Phys. Lett.* **98A**, 12–14; 1983).

Quantum mechanics assigns a probability amplitude to a particular transition sequence from an initial atomic state to a final state. This amplitude has contributions from all available routes provided the same initial and final states are involved. The probability of a transition is the absolute square of the contributing amplitudes and exhibits interferences between the participating routes. These interferences are well known in spectroscopy and are the basis of so-called quantum beats and of asymmetric line-shapes in autoionization in which an excited atomic state can decay by emission of an electron (not a photon).

The fundamental theory of autoionization due to Ugo Fano (*Phys. Rev.* **124**, 1866–1877; 1961) shows that interference takes place in the excitation of an autoionizing resonance: the atomic ground-state population can be excited directly to produce an outgoing electron, or can be excited indirectly to an upper level from where it decays to the ejected electron state and these two routes interfere. In many cases the interference generates asymmetric absorption line shapes which at certain frequencies are zero because of the destructive interference of the two contributing pathways. These zeros, or 'Fano windows', are responsible for the absence of absorption at such frequencies.

Harris exploits such interference to suppress absorption in his notional population-inversion-free laser. The surprise is that it does not suppress the

emission. The emission profile from the upper level(s) (the autoionizing state(s)) does not involve the same Fano interference. In emission we are interested in the pathways from the excited atomic state (not from the ejected electron) back to the atomic ground state and a different route is taken. The lack of symmetry between absorption (which experiences interference suppression) and emission, means emission can take place at suitable frequencies without any risk of back-transitions absorbing the stimulated photons. So inversion is not required.

Nonlinear optical-frequency mixing experiments performed several years ago (J. A. Armstrong & J. J. Wynne *Phys. Rev. Lett.* **33**, 1183–1185; 1974), which I

EVOLUTION

Neotropical African bees

Robert E. Page Jr

DESCENDANTS of African honey bees that were imported into Brazil in 1956 have successfully colonized most of South and Central America and are now spreading throughout Mexico. The spread of this feral 'Africanized' population has been followed and documented for more than 25 years, but the genetic and behavioural mechanisms behind its great success and the maintenance of its many African phenotypic characteristics have eluded explanation. In two papers in this issue, Hall and Muralidharan on page 211¹ and Smith *et al.* on page 213², show that the feral Africanized population that is now establishing itself in Mexico shares a common maternal heritage throughout Central and South America — and that heritage is African.

In 1956, Warwick Kerr imported queen honey bees from South Africa into southern Brazil³. His objective was to produce a strain of honey bees that had the good management characteristics of European subspecies but was tropically adapted like those of Africa. European bees are easily managed but not very productive in Brazil's tropical climate. African bees, although productive in the tropics, are difficult to manage, prone to severe stinging behaviour and therefore undesirable to commercial beekeepers. Kerr established a bee-breeding and stock-testing programme with these imported queens; but by 1963 vicious, unmanageable African bees and their hybrids comprised an estimated 70 per cent of the total honey-bee population of the state of São Paulo, an area slightly larger than Great Britain³.

have reviewed (*Comments at. mol. Phys.* **15**, 193–214; 1984), already indicated that absorption suffers from destructive interference and Fano windows, whereas the emission of optical harmonics does not.

Harris's predictions take this kind of effect into account in the dynamics of active laser material. To observe such interference requires interfering pairs of autoionizing levels which must decay to a common outgoing electron state and these are known to occur (for example in the Stark effect, induced by static electric fields, in highly excited atoms). Whether it constitutes more than a fascinating example of quantum interference or can be the foundation of new laser devices remains to be seen. □

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How this predominantly African population became established in an area that had had European subspecies of bees for more than 100 years has remained an enigma. One explanation is that 26 African queens escaped from their hives in 1957. These pure Africans were mated to African drones and reputedly became the focus of the process of Africanization. From this initial focus, there are strongly differing opinions⁴ about how African traits were initially maintained in an area where the reproductive progeny of these 26 queens should have had greater chances to mate with bees of European ancestry, thereby 'diluting' the African genes in a pool of Europeans. Opinions also differ about how this population now seems to resist introgression of genes from the commercial and feral European bees it has encountered during its spread.

Rinderer and colleagues at the US Department of Agriculture—Agriculture Research Service propose that the process of Africanization did then and does still involve hybridization of the feral and commercial bees⁵. As a consequence, these bees constitute a hybrid swarm and should be called 'Africanized' rather than 'African'. The authors of both papers in this issue^{1,2} maintain, as did one of them, O.R. Taylor, in a paper in 1985⁶, that the African population has remained distinct from the European population through intersubspecific competition and reproductive isolating mechanisms. Hall and Muralidharan¹ speculate that superior-performing African mitochondria provide better metabolic machinery for long-distance dispersal, or that intersubspecific

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A swarm of Africanized hive-bees, *Apis mellifera scutellata* — or plain African? incompatibility of mitochondrial and nuclear DNA make hybrids less fit. These authors maintain^{1,2} that European bees have not contributed significantly to the feral, Africanized gene pool and it, therefore, should be called 'African'.

The reported demonstration of high frequencies of African-type mitochondria in honey-bee populations in South and central America, together with Mexico, suggests an unbroken African maternal lineage for most feral bees observed. The observed low frequency of European-type mitochondria seems to support the hypothesis that colonies with queens of European descent have been unsuccessful in competition with colonies having queens and workers of direct African maternal descent^{1,2}. But neither report constitutes a true population study of the processes of Africanization. Assuming that different colonies were sampled in the two studies, a total of 92 Africanized colonies were sampled from four locations on two continents. Incidentally, two of these locations have unconfirmed reports of separate introductions of bees from South Africa: Venezuela in 1974⁷ and Mexico in 1976⁸.

Even though the data are convincing that feral bees in Mexico have a direct maternal lineage with those introduced

into Brazil, population studies of the co-occurring distributional patterns of mitochondrial and nuclear DNA throughout Mexico and South and Central America are still needed. Only these kinds of studies will ultimately demonstrate the populational processes of Africanization. Analyses of morphometric traits believed to be polygenic and nuclear, and of feral, presumably Africanized, honeybees from Acarigua, Venezuela (one of the areas demonstrating African-type mitochondria) show worker phenotypes intermediate in character between the small-sized African subspecies and the larger-sized Europeans⁹. These characters are not different from those previously reported that have been collected throughout South America¹⁰, suggesting a spreading population of hybrid origin mainly, but not exclusively, of African descent.

According to Taylor⁶, there were only 30,000–40,000 colonies of European bees in Venezuela in 1976, just before the beginning of Africanization, whereas today there are probably 1–2 million feral colonies of African bees. If this is true, special mechanisms of subspecific hybrid inferiority, limited gene flow, or competitive interactions between African and European populations do not need to be invoked to explain the absence of European mitochondrial DNA in the 22 Venezuelan colonies reported in these two studies^{1,2}. European mitochondria should be very rare if the feral population resulted from the expansion into Venezuela of a very large, rapidly growing population of African maternal descent. Future studies in Mexico, with its larger commercial honey-bee population and growing feral population, should yield exciting information if elements of both mitochondrial and nuclear DNA are studied and reported concurrently.

What still eludes explanation are the initial conditions in southern Brazil 30 years ago that resulted in the establishment of a large, mainly African feral population of honey bees that has spread as a continuous maternal line across two continents. The determination of these conditions may be more suitable for historians than geneticists, but is critical for a complete understanding of this truly extraordinary biological phenomenon. □

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Dry drinking

SHOULD you find yourself adrift at sea in an open boat, don't drink the sea water. Its salt has to be disposed of later in a much larger volume of sweat or urine, so you always make a loss on the deal. Many solar stills and ion-exchange devices can make sea water drinkable, but Daedalus now has a new approach. Human skin, he points out, is itself a semipermeable membrane. Water and small molecules can diffuse through it, but electrolytes cannot. Accordingly, it should be possible to imbibe pure water from the sea by reverse osmosis, by pressurizing sea water against your skin.

At first Daedalus imagined some simple gadget like a blood-pressure cuff with a porous patch on it, pumped up with sea water against the arm. But he soon realized that this would be a recipe for complete strangulation. Over 20 atmospheres of pressure are needed to overcome the osmotic-pressure difference between sea water and human blood plasma.

The answer is to apply the pressure over numerous tiny areas of skin, each so small that even 20 atmospheres does not represent excessive local force. DREADCO's physiologists are devising a skin patch with an input pipe leading to many closely spaced little channels indented into the patch surface. The regions between the channels are covered with sticky, tenacious, biocompatible cyanoacrylate resin. When the patch is peeled off its support film and stuck onto the skin, the cyanoacrylate glues it permanently in place. A pump on the input pipe can then force seawater into the tiny channels under the required hydraulic pressure, with no strangulation of blood flow and little local discomfort.

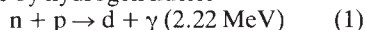
But skin is so impermeable that even an osmotic patch at 30 atmospheres, and covering the entire body, could hardly supply enough water to sustain life. Fortunately, dilute detergent solutions make the skin dramatically more permeable. If you add a little detergent to your sea water, a patch about 30 centimetres across should keep you adequately hydrated at the cost of steady pumping. The osmotic patch will probably be most convenient on the back or abdomen. Once in position, it cannot be removed, but will ultimately come away with the renewal of the top layer of skin.

Water that is merely dirty or contaminated, but with a lower osmotic pressure than human fluids, should be imbibed even more easily: spontaneously, in fact. Just add a little detergent, and sit in it! This suggests a new strategy for those who distrust the public water supply, and also those whose principles forbid them to drink alcohol. DREADCO's high-alcohol detergent bath salts will let them get virtuously drunk without a drop passing their lips.

David Jones

Problems with the γ -ray spectrum in the Fleischmann *et al.* experiments

SIR—Fleischmann, Pons and Hawkins¹ recently announced the observation of significant heating in their cold-fusion experiments, a result that they attribute to copious fusion reactions. As compelling evidence that fusion had occurred, they reported the observation of the 2.22-MeV γ -ray line that originates from neutron capture by hydrogen nuclei^{2,3}



(Here d represents a deuteron.) They contend that the neutron in reaction (1) is generated by the reaction



and conclude, therefore, first that the 2.22-MeV γ -ray confirms that the fusion process (2) is occurring, and second that a neutron production rate of the order of 4×10^4 neutrons s^{-1} is derivable from

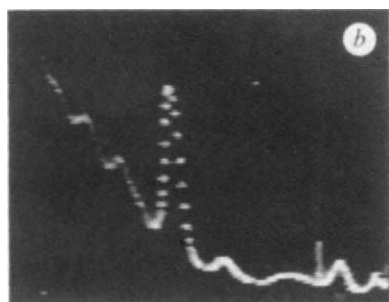
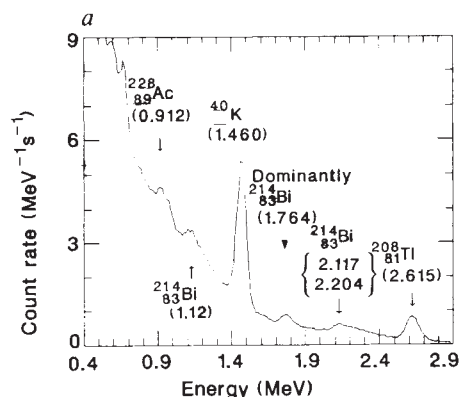


FIG. 1 *a*, The γ -ray background spectrum measured with a 3 in. $\times$ 3 in. NaI(Tl) detector at MIT. Some important terrestrial γ -ray lines have been identified in this figure^{6,7,12}. (As explained in ref. 12, the immediate parent of the final decay product is identified. For example, ${}^{40}\text{K}$ β^+ decays into an excited nuclear state of ${}^{40}\text{Ar}$, which actually then emits the 1.460-MeV photon discussed in the text.) The spectrum is averaged over an 84-hour run. *b*, The γ -ray spectrum shown on television by Fleischmann *et al.* The main characteristics of the two spectra are similar; one can also tell that the two detectors have comparable spectral resolution. In *b*, note the curious structure at about 2.5 MeV and that beyond the ${}^{208}\text{Tl}$ peak (2.61 MeV), which appear to be artefacts. (The spectrum can also be obtained from KSL-TV in Utah (M. Hawkins, personal communication).)

their γ -ray signal rate. They further state that most of the heat generation occurs not through process (2), but through a hitherto unknown nuclear-fusion process.

Here we focus solely on the identity of the reported γ -ray line, which we shall henceforth call the signal line. We argue that the claim of Fleischmann *et al.* to have observed the 2.22-MeV line characteristic of reaction (1) is unfounded. We do so on the basis of three quantitative considerations: (1) that the linewidth is a factor of two smaller than their instrumental resolution would allow; (2) that a clearly defined Compton edge⁴, which should be evident in their published data at 1.99 MeV, is not in fact present; and (3) that their estimated neutron production rate is too large by a factor of 50. In addition, from a consideration of the terrestrial γ -ray background, we argue that their purported γ -ray line actually resides at 2.5 MeV rather than 2.22 MeV. These conclusions are, in part, based on our studies of neutron capture by hydrogen, using a neutron source submerged in water. These measurements allow us to compare the results of Fleischmann *et al.* directly with a controlled experiment.

We measured terrestrial γ -ray background spectra in order to compare our detector characteristics with those of Fleischmann *et al.* Figure 1*a* shows a typical terrestrial γ -ray background spectrum obtained with a 3 in. $\times$ 3 in. NaI(Tl) crystal spectrometer system (see ref. 5 for details). The main features of the background spectrum are quite similar throughout the

terrestrial environment^{6,7}. Fleischmann *et al.* showed a similar γ -ray spectrum on television (Fig. 1*b*). (We believe that we have viewed all the cold-fusion γ -ray spectra that have been shown on KSL-TV (Utah) up to 19 April. This information was obtained from Utah News Clips, Inc., Utah. As far as we can tell, all spectra are identical to that of Fig. 1*b*.) This spectrum was obtained in the course of the Fleischmann *et al.* experiments (M. Hawkins, personal communication). Their spectrometer system consisted of a Nuclear Data ND-6 portable analyser with a 3 in. $\times$ 3 in. NaI(Tl) crystal (ref. 1 and M. Hawkins and R. Hoffmann, personal communications). A $\frac{3}{8}$ -in.-thick Pb annulus encompassed the scintillator. It is clear from Fig. 1*a* and *b*, and particularly from the ${}^{40}\text{K}$ (1.46 MeV) and ${}^{208}\text{Tl}$ (2.61 MeV) lines, that our resolution is comparable to or better than that of the spectrometer used by Fleischmann *et al.*, a point to which we return later.

In the interval 1.46–2.61 MeV, the energy resolution of a NaI(Tl) spectrometer, which determines the γ -ray linewidth, can be well described by the formula^{8,9}

$$R(E) = \frac{\Delta E}{E} \approx R(E_n) \sqrt{E_n/E} \quad (3)$$

Here ΔE is the full width at half maximum (FWHM) of the line, E is the energy of the photon and $R(E_n)$ is the measured 'reference' resolution at energy E_n . $R(E_n)$ can be accurately determined using a ${}^{60}\text{Co}$ source (that is, the ${}^{60}\text{Co}$ line at 1.33 MeV), or it can be fairly well approximated by the ${}^{40}\text{K}$ decay line at 1.46 MeV. (From Fig. 1*b*, the ${}^{40}\text{K}$ decay line allows one to estimate Fleischmann *et al.*'s resolution as $\sim 8\%$.)

TABLE 1 Comparison of energy resolutions of the γ -ray spectrometers

(a) Resolution of MIT spectrometers					
Energy (MeV)	1.17 ${}^{60}\text{Co}$	1.33 ${}^{60}\text{Co}$	1.46 ${}^{40}\text{K}$	2.22 $n(p,\gamma)d$	2.61 ${}^{208}\text{Tl}$
Origin					
Natural background			0.055		0.043 (0.041)
${}^{60}\text{Co}$	0.056	0.051			
Pu/Be neutron source				0.05 (0.045)	
(b) Resolution of the Fleischmann <i>et al.</i> spectrometer ¹					
Energy (MeV)	1.33 ${}^{60}\text{Co}$	1.46 ${}^{40}\text{K}$	2.22 $n(p,\gamma)d$	2.61 ${}^{208}\text{Tl}$	
Reference					
Hoffman*	0.056	0.065			
TV news†		~ 0.08			
Ref. 1 (errata)			0.025 (0.053)		~ 0.05 (0.049)

The resolution is defined as the full width at half maximum (FWHM) divided by the peak energy. Numbers in parentheses are predicted values based on the detector resolution at 1.46 MeV (see text). In *b*, the prediction is based on the resolution value (0.065 at 1.46 MeV) provided by R. Hoffman (personal communication).

*R. Hoffman (personal communication).

†Derived from images of the televised news broadcasts.

Table 1 lists the resolution data for our detectors and for that of Fleischmann *et al.*.

We now compare the signal line of Fleischmann *et al.* (Fig. 1a of ref. 1 (errata), shown as Fig. 2 here) with our measured spectrum obtained from the experiments on neutron capture by hydrogen (Fig. 3 here, and Fig. 4 of ref. 5). In these experiments, a Pu/Be neutron source was placed in a water tank. ^{239}Pu emits energetic α -particles, which produce neutrons through (α, n) reactions with Be (refs 4,9). The neutrons are thermalized in water, and we observe the emitted neutron-capture γ -rays with our spectrometers. The measured resolution at 2.22 MeV is $\sim 5\%$ (Table 1a), and is reasonably well predicted by equation (3). As a consequence, this calls into immediate question the identity of Fleischmann *et al.*'s signal line as a γ -ray line. Specific-

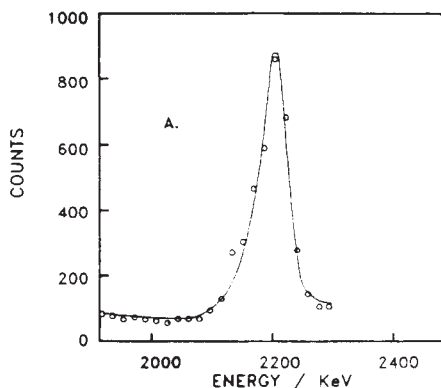


FIG. 2 A reproduction of the purported 2.22-MeV γ -ray signal line of Fleischmann *et al.* (Fig. 1a of errata to ref. 1). The resolution, based on the linewidth, is about 2.5%. With such resolution, one would expect to see a clearly defined Compton edge at 1.99 MeV. No edge is evident. Also, a resolution of 2.5% is inconsistent with their spectral resolution. Furthermore, we argue that the signal line may reside at 2.5 MeV, not at 2.22 MeV as is claimed by Fleischmann *et al.* and depicted here.

ally, Fig. 2 shows the signal line to have a resolution of 2.5%. This is about a factor of two smaller than that predicted by equation (3) on the basis of the known resolution (Table 1b) from either the ^{40}K decay line (1.46 MeV) or from the ^{60}Co source (1.33 MeV) (R. Hoffman, personal communication). But we know from Table 1 that the spectrometer used by Fleischmann *et al.* has a resolution that is at best comparable to our own for the entire region from 1.46 to 2.61 MeV (see also Fig. 1), so it is inconsistent that their linewidth at 2.22 MeV is a factor of two below the predicted value.

There is a second crucial inconsistency with the published signal line (Fig. 2). If we assume a resolution of 2.5% at 2.22 MeV, then there should be a clearly defined Compton edge⁴ at 1.99 MeV. For example, in Fig. 3 the Compton edge is evident even for our measured resolution

of only 5%. For a resolution of 2.5%, the definition of the Compton edge would be distinctly sharper. The lack of a Compton edge at 1.99 MeV for the signal line therefore negates the conclusion of Fleischmann *et al.* that they have observed the 2.22-MeV γ -rays from neutron capture by hydrogen.

We also point out that in our (Pu/Be) neutron-capture experiments, a conspicuous e^+e^- annihilation single-escape peak exists at 1.71 MeV (Fig. 3), as well as a double-escape peak at 1.20 MeV. (The full spectrum from the Pu/Be experiment, as well as the background spectrum, can be found in ref. 5.) Such features unambiguously identify the primary γ -rays as having an energy of 2.22 MeV, and are a necessary consequence of the physical processes of detection of γ -rays in a finite-sized NaI scintillator.

Based independently on both their γ -ray and neutron measurements, Fleischmann *et al.* claim to have observed a neutron production rate of $\sim 4 \times 10^4$ neutrons s^{-1} (ref. 1). This claim is clearly inconsistent with their γ -ray signal line, for the following quantitative reasons. The Pu/Be neutron source used in our experiment is absolutely calibrated to within 10% of 1.5×10^6 neutrons s^{-1} (ref. 10 and MIT Reactor Radiation Protection Office). In obtaining the data in Fig. 3, we used an experimental setup similar to that of Fleischmann *et al.* (ref. 1; televised broadcasts; and M. Hawkins and R. Hoffman, personal communications). Our Pu/Be source was submerged 6 in. into a large water tank. The rate at the 2.22-MeV peak, after subtracting the background continuum, is about 1.4×10^3 $\text{MeV}^{-1} \text{s}^{-1}$ (see Fig. 3). Scaling this rate to a neutron source of 4×10^4 neutrons s^{-1} (the level given by Fleischmann *et al.*), and integrating over the linewidth, gives a total 2.22-MeV γ -ray rate of about 4.5 counts per second. This value is a factor of 50 times higher than the rate that would be calculated on the basis of the results in Fig. 1a (that is, 0.081 counts per second). (Fleischmann *et al.* state that their neutron count rate is measured with a BF_3 neutron counter over a $0.4 \text{ mm} \times 10 \text{ cm}$ Pd cell, and that the γ -ray measurement is over a $0.8 \text{ mm} \times 10 \text{ cm}$ Pd cell¹. If the total reaction rate is proportional to the volume of Pd rod, as they state, the inconsistency in the reported neutron rate is by a factor of 200 rather than 50.) While differences in rates of a factor of two might possibly be explained by geometrical considerations, a factor of 50 is inexplicable.

A further point concerning the identification of the signal line is the precise value of the energy at which the peak occurred. From Fig. 2, the background in the neighbourhood of the peak is seen to be ~ 80 counts per channel, a level that corresponds to ~ 400 counts per channel for a 48-hour accumulation time (the data in

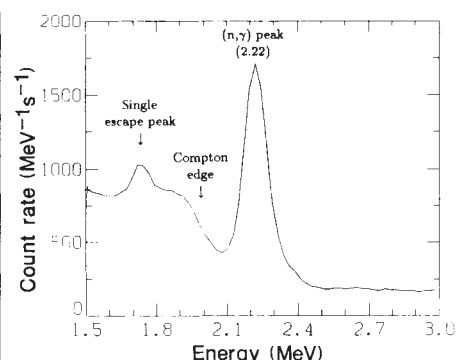


FIG. 3 The γ -ray spectrum measured by a 3in. $\times$ 3in. NaI(Tl) spectrometer during a neutron-capture-by-hydrogen experiment using a (Pu/Be) neutron source submerged in water. Because of the finite size of the crystal (which is identical to that of Fleischmann *et al.*¹), we also see an escape peak²⁻⁴ and, of particular importance here, the Compton edge⁴. In this figure, the digitization energy width is 0.024 MeV per channel. The full Pu/Be and background spectra are shown in Fig. 4 of ref. 5.

Fig. 2 were accumulated for a period of 10 hours¹). On the other hand, in the Utah measurements of terrestrial γ -ray background, the level in the vicinity of the 2.22-MeV feature was found to be $\sim 4,000$ counts per channel (R. Hoffman, personal communication). The only relevant part of the entire γ -ray spectrum (between 1.46 and 2.61 MeV) in which the background was as low as 400 counts was at an energy in the vicinity of 2.5 MeV (R. Hoffman, personal communication). Thus, we argue that the peak in the spectrum shown in Fig. 2 may be at 2.5 MeV, not at 2.22 MeV.

The importance of properly identifying the energy of the feature claimed by Fleischmann *et al.* can hardly be over-emphasized. Thus, it is extremely unfortunate that they chose to display only the energy range 1.9–2.3 MeV in their pub-

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lished Fig. 1a, thereby not providing the supporting evidence of the ^{40}K (1.46-MeV) and ^{208}Tl (2.61-MeV) features which must be present in their spectra in order for their identification to be correct.

Therefore, although Fleischmann *et al.* may have observed a change in their γ -ray spectra that bears some relation to detector location, we conclude that it is unrelated to the 2.22-MeV neutron-capture γ -rays, and that it is also unrelated to the background ^{214}Bi line (2.20 MeV; Fig. 1a), as has been suggested elsewhere¹¹. We can offer no plausible explanation for

the feature other than it is possibly an instrumental artefact, with no relation to a γ -ray interaction.

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Fusion in from the cold?

SIR—Recent experiments involving the loading of deuterium (D) into palladium¹ via a 0.1 M LiOD/D₂O electrolyte may have created something not encountered in the absence of current flow. The voluminous body of older work on electrolytic D–Pd loading is based on thin wires or foils of high aspect ratio which allow only small voltage differentials. But the reported chemical potential across a thick deuterated palladium negative electrode could give rise to metastable solid phases alien to the existing equilibrium Pd–D phase diagram. The theoretical existence of palladium analogues to hydrogen-rich² compounds like Li_2ReH_6 is as tantalizing as the limit of solid solubility of D in Pd is finite. And beyond PdD_3 (by analogy to the known compound Li_2Pd) the thermodynamic distinction between the higher palladium deuterides and metallic deuterium grows dim. The mechanism of their formation might involve the intersection of high deuteron mobility grain boundaries with the Cottrell clouds of H and D that decorate dislocations in many transition metals.

A small mass of (mostly) deuterons transforming into molecular deuterium as they recapture electrons previously delocalized into the S- and D-bands of palladium could release roughly 1 MJ mol⁻¹. Apart from explaining the electrode meltdown and vaporization reports¹ (although this could be due to lithium's remarkable ability to lower the melting point of palladium), this could raise local temperatures to very high values.

Could the so-called 'cold fusion' environment in fact involve local temperatures of greater than 10⁵ K generated by the detonation of deuteron clusters (R. G. Gordon, personal communication) or of a metallurgical precipitate, coarse or fine-grained, of a high D/Pd ratio intermetallic compound inside a deuterated palladium electrode? The energy of reassociation of electrons and deuterons is roughly 1 Rydberg (13.6 eV) minus the work-function of Pd (4.9 eV); to this must be added the roughly 4.7 eV liberated when two deuterium atoms pair. Obviously, these energies must be scaled down to compen-

sate for the difference between reactions in free space and the solid state. Nevertheless, the 20 eV energy of formation of D₂ from deuterons could thus produce hot and highly compressed deuterium plasma bubbles of small ($>0.01\mu\text{m}$ – $<100\mu\text{m}$) size. As to the objection that the surrounding cool metal will quench these bits of pale fire in a nanosecond or so, I believe it answers a serious question: how come the Fleischmann and Pons¹ claimed neutron yield is 9–14 orders of magnitude short of their claimed heat flux? At present, one can only speculate as to by how many orders of magnitude a reflected spherical shock front might raise the temperature and pressure of the D-plasma.

There is another ramification to the notion of nano-novas flashing out of per-

deuteride grains decorating the grain boundaries of electrochemically overwrought palladium negative electrodes. The explosion of such precipitated grains will, above a critical radius, generate cracks in the adjacent metal. It has been postulated (G. Chapline, personal communication) that the surfaces of such cracks, as they open, could host a field gradient or a propagating array of plasmons down whose wake field a deuteron could accelerate. This presumes that the bulk Pd–D system has reached 1:1 stoichiometry and thus been restored to long-range order; this condition is very far from the disorderly and anharmonic state of Pd–D during the early stages of electrolytic D-loading. In this model some thousands of unit cells of travel would suffice to yield keV deuterons. So the propitiated shade of Rutherford may yet countenance not-so-cold fusion.

It has been noted in a News and Views article in the 27 April issue³ that neutrons have been observed when cracks are generated in crystals of lithium deuteride⁴.

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Mössbauer cancer therapy doubts

SIR—Mills *et al.*¹ present data to support the view that a dose of 10⁻⁵ Gy of 14.4-keV X-rays can ablate a population of malignant cells containing $^{57}\text{Fe(III)}$ · bleomycin. They suggest that such a regime may have the potential for the low-dose sterilization of superficial human tumours.

This is unlikely on simple physical grounds, basically because only a small fraction of the exposed cells will have received any energy deposition at all. The proportion of cells that would receive one or more energy depositions, assuming the statistical independence of such events, is obtained from the Poisson distribution and is $(1 - e^{-n})$; here n , the average number of energy depositions occurring in the sensitive site, is given by D/z_{IF} , where D is the absorbed dose and z_{IF} is the so-called 'frequency-averaged specific energy per event' — or simply, the mean energy per unit mass deposited by single energy deposition events in the sensitive site in the cell². This mean specific energy will be similar, irrespective of whether the photon is ultimately absorbed in a photoelectric event or in a Mössbauer absorption (it would be slightly larger in the latter case); it has been measured for 12-keV photons in a tissue-equivalent material in spherical sites of various volumes³.

Appropriate volumes for consideration are those of typical human cell nuclei (100–1,000 μm^3)⁴ or, perhaps more relevantly, the volume of nucleotides in the mammalian nucleus ($\sim 3\mu\text{m}^3$)⁵. For volumes of 250 and 3.5 μm^3 , for which measurements have been made³, the corresponding values of z_{IF} are 4×10^{-3} Gy and 0.14 Gy. These numbers yield a probability of about 2.5×10^{-3} that a 250 μm^3 volume of cell nuclei will be subject to at least one energy deposition, and a corresponding probability of 7×10^{-5} for a 3.5 μm^3 volume of nucleotides. Thus, only about 1 in 400 cells (250 μm^3 volume) or 1 in 14,000 cells (3.5 μm^3 volume) would receive any energy deposition at all if exposed to a dose of 10⁻⁵ Gy.

To sterilize even a small tumour containing about 10⁸ cells requires an appropriately small probability (less than 10⁻⁹) that any cell will receive no energy-deposition events. Thus, an average number of energy depositions per cell of

1. Mills, R.L. *et al. Nature* **336**, 787–789 (1988).
2. *Microdosimetry ICRU Rep. 36* (ICRU, Bethesda, 1983).
3. Kliauga, P.J. & Dvorak, R. *Radiat. Res.* **73**, 1–20 (1978).
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5. Brenner, D.J. *Radiat. Envir. Biophys.* **27**, 189 (1988).
6. Goodhead, D.T., Thacker, J. & Cox, R. *Phys. med. Biol.* **26**, 1115–1127 (1981).

about 20 would be needed, requiring doses of 0.08 Gy for a nuclear volume of $250 \mu\text{m}^3$, or 2.8 Gy for a nucleotide volume of $3.5 \mu\text{m}^3$. Such numbers are far larger than those proposed by Mills *et al.*

Finally, an explanation for the ablation effects seen by Mills *et al.*¹ may, in part, be that about 1 in 10 cell nuclei in their exposed monolayer would have received energy depositions from interactions with

6.5-keV iron X-rays from the Mössbauer source. Such low-energy X-rays have a high relative biological effectiveness⁶.

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Surface-plasmon microscopy

SIR—We recently introduced surface-plasmon microscopy (SPM)¹ as a new optical imaging technique with specific advantages for low-contrast thin-film samples like a lipid monolayer transferred from the water–air interface to a solid substrate. We claimed² that other light-microscope techniques, such as phase-contrast or Nomarsky microscopy, fail if one wants to see the coexistence of expanded and condensed domains of a single monolayer transferred at its phase transition pressure without the use of fluorescence labels to enhance the contrast between the two phases^{3,4}. Sheppard *et al.*⁵ dispute this claim but rather than comment on their somewhat surprising results, obtained with a monolayer film of ω -tricosenoic acid and scanning optical microscopy, we prefer to substantiate our claim that only the high resonance contrast² achievable in SPM allows one to study thin-film samples of very low thickness and/or index variations.

A monolayer of dimyristoylphosphatidic acid (DMPA) forms a quasi-two-dimensional system, known to undergo a lateral

pressure-induced first-order phase transition from an expanded liquid-analogue to a condensed solid-analogue phase. The two phases can coexist over a broad density range (Fig. 1a). If a suitable fluorescent dye is added to the lipid layer

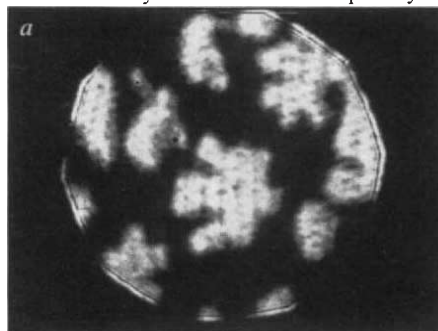


FIG. 2 SPM picture of a DMPA monolayer without dye, transferred to the solid support at the lateral pressure where condensed and expanded domains coexist. Angles of incidence, θ_1 , were a, 47.20° and b, 47.70° .

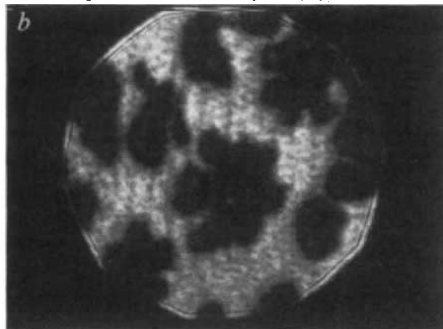
the coexistence can be observed directly: because the dye is less soluble in the condensed phase it is squeezed out of the growing crystalline domains^{3,4}, which appear dark in a picture made by fluorescence microscopy (Fig. 1b).

SPM permits the imaging of such a monolayer without the need for a fluorescent label. The experimental set-up is fully described in ref. 1, but the essence of the method is that a p-polarized beam of laser light of wavelength λ is applied, by means of a prism, at an angle θ_1 to a metal–dielectric interface, where it excites surface plasmons (PSP) whose wavevector fulfils the momentum matching condition $k_{sp} = k_{\text{photon}}$, where k_{photon} is the parallel component of the photon wavevector. The energy–momentum relation for the PSP, which gives k_{sp} , is determined by the optical architecture of the interface and, in particular, by the thickness and the dielectric functions of the metal and the coating, respectively. Any small local change in the thickness or the real and/or imaginary part of the complex index of refraction of a thin coating therefore changes this resonance condition slightly, which gives rise to the high contrast between different coatings that can be achieved with SPM.

The PSPs that are reflected, diffracted or scattered by the interfacial (coating) inhomogeneities couple out via the prism and are Fourier-backconverted (by a simple lens in our case) to form an image

in real space. This is demonstrated in Fig. 2 for a DMPA monolayer transferred onto a gold substrate without any dye added. The interference pattern in the bright areas of each picture arise from insufficient spatial filtering.

Despite the lower lateral resolution (compared with the fluorescence microscope picture), which is — for our set-up — about $5 \mu\text{m}$ (ref. 7), it is evident that the same dendritic growth figures can be identified as in the presence of the dye. In addition, however, SPM enables us to perform a more quantitative analysis of the optical properties of the coexisting domains, as we will report in detail elsewhere (W.H. and W.K., in preparation). Given the thicknesses of the condensed and expanded monolayer ($d_1 = 2.25 \text{ nm}$



and $d_2 = 1.65 \text{ nm}$, respectively)⁸ we obtain indices of refraction $n_1 = 1.51$ and $n_2 = 1.304$ for the two different phases. The lower value for the fluid (amorphous) domains is consistent with a remarkably low electron density found for this phase by X-ray data (C. Helm and H. Möhwald, personal communication) but may, in addition, indicate a further expansion of these areas on deposition to the substrate.

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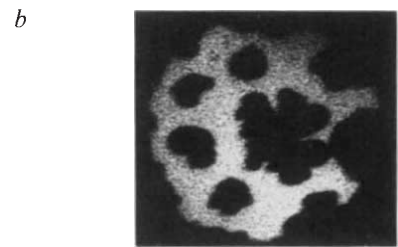
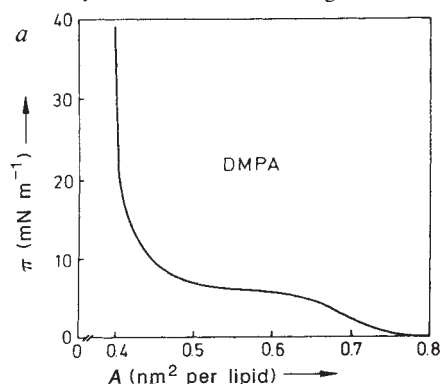


FIG. 1 a, Pressure–area (π – A) isotherm of DMPA on pure water, pH 5.8, $T = 25^\circ\text{C}$. b, Fluorescence microscope picture of a DMPA monolayer at the water surface. Dark areas, crystalline domains with a low solubility of the fluorescence dye used.

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6. Gaines, G.L. Jr *Insoluble Monolayers at Liquid-Gas Interfaces* (Interscience, New York, 1966).
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Erratum

SIR—In the Scientific Correspondence on cold fusion in the 27 April issue of *Nature* (p. 711) by A. J. McEvoy and C.T.D. O'Sullivan, the first author's name is misspelled and the last sentence of the first paragraph should read: "If this number could be increased by a factor of 10....". □

Seeing things differently

Brian Rogers

James J. Gibson and the Psychology of Perception. By Edward S. Reed. Yale University Press: 1989. Pp.348. \$32.50, £25.

FEW psychologists have provoked as much controversy as James Gibson did (and still does) with his ideas about the nature of perception. Comments have ranged from Hinton's mildly teasing "Is Gibson being silly?", with respect to the claim that perception is direct, to Sutherland's remark that "Gibson did for perception what Skinner did for animal learning: he handicapped a generation of workers by his blinkered and oversimplified approach". Reed's biography of Gibson is unlikely to change such strongly held opinions. But for anyone interested in the historical background and development of Gibson's ideas from the 1920s until his death in 1979, the book will make fascinating reading. Moreover, it complements Reed and Jones's *Reasons for Realism*, an earlier edited collection of Gibson's published and unpublished papers.

The structure of the biography is roughly chronological — Reed describes the academic and political influences on Gibson as a conventional experimental psychologist in the 1930s, and then proceeds to show how his ideas about perception were gradually transformed into what most would regard as a radically different theory. The book provides interesting insights into the significance Gibson attached to the results of his well-known experiments on adaptation to curved lines in the 1930s. The fact that adaptation could be demonstrated in a dimension (shape) that philosophers had previously regarded as 'primary', prompted Gibson to question the accepted doctrine of sensationism: "What it [the experimental result] does imply is that the distinction between primary and secondary qualities may well be unfounded. If this is true it would seem to follow that the common assumption of a 'world of physics' separable from the 'world of experience' is also invalid" (p.45).

Two additional influences on Gibson's thinking, according to Reed, were the economic climate of the Depression in the 1930s, when Gibson was at Smith, and the political climate of the McCarthy period after he had moved to Cornell. In the 1930s and 1940s Gibson was involved in various 'radical' groups, including the Academic Freedom Committee of Society for the Psychological Study of Social Issues and the American Federation of Teachers. These activities were probably responsible for the FBI investigations into Gibson's 'communist affiliations' and his subsequent loss of security clearance for

Air Force research, although no charges were ever brought against him. Reed says that "The experiences of the 'great fear' helped shape Gibson's transformation from a mainstream psychologist into a scientific dissident" (p.112). The book also contains an interesting chapter describing Gibson's thinking and writing about social psychology which were subsequently abandoned in the early 1950s. The academic influences on Gibson were many and



James J. Gibson — "one of the most fascinating figures of twentieth-century psychology".

varied — from his teachers Langfeld and Holt at Princeton in the 1920s, to the Gestalt psychology of Koffka at Smith during the 1930s, and eventually to the 'European' influence of Kohler, Michotte and Johansson in the 1950s.

The landmarks in Gibson's career are without doubt his three books — *The Perception of the Visual World* (1950), *The Senses Considered as Perceptual Systems* (1966) and *The Ecological Approach to Visual Perception* (1979). The first, which was the result of his work in the US Army Air Forces' Psychological Test Film Unit during the Second World War, contained two principal ideas. First, Gibson stressed the importance of gradients in visual stimulation; second, he provided the first extensive discussion since Helmholtz of the potential importance of flow in the retinal image, created when an observer moves with respect to his surroundings. Reed suggests that Gibson's earlier work on adaptation to curved lines "made him doubt that any simple constant values of stimulation were useful in perception" and consequently influenced him to look

for what he later referred to as invariants or 'constant patterns of variation' (p.82) such as those found in the retinal gradient of texture density produced by the ground surface. Moreover, Gibson pointed out that the same surface would generate gradients of retinal motion for a moving observer which, he said, provided a "structure specific to locomotion" (p.84).

What he had identified, albeit using less rigorous and non-mathematical terms, is what David Marr described 30 years later as 'constraints' imposed by the physical structure of the world we live in. Gibson's own attempts to determine the effectiveness of texture gradients were, however, largely unsuccessful, and (contrary to many textbook accounts) he later doubted whether static gradients, by themselves, were capable of specifying the slants of

surfaces. The transformation of the 'optic array' produced by the moving observer, on the other hand, was to remain a key idea.

Gibson's second book, *The Senses Considered as Perceptual Systems*, was notable for its rejection of the traditional way of describing the input for perception in terms of stimuli and the formation of a retinal image. Instead, Gibson proposed that perception could only be understood in terms of the information available (in the transforming optic array) to an active perceptual system, rather than in terms of stimuli (energy) capable of exciting the sensory receptors. This idea was later expressed in a characteristically provocative style: "There is no stimulus for vision. [The] retinal image is irrelevant for functional vision" (p.283).

In the same book the concept of an 'affordance' was introduced — the term was defined as "what the world offers" the perceiver, in an attempt to show that not only physical properties but also meaning could be specified by the way light is structured. This idea was crucial to

Gibson's claim that the sensory input does not need to be supplemented by results of mental processes such as inference, which most psychologists assumed and still assume to be the case. Tree stumps afford sitting on, Gibson argued, and (more radically) "postboxes afford letter mailing" (p.139). At this point most psychologists either give up in despair or, like Foder and Pylyshyn, accuse Gibson of using the term so loosely as to make it meaningless.

How can one begin to evaluate such an extensive and controversial legacy? Reed, who is clearly very sympathetic to Gibson's ideas, explicitly refrains from an objective assessment on the grounds that the book "is a biography, not a critical essay" (p.7). That is a great pity, in my view, although quite consistent with Gibson's own characteristic style of simply ignoring "traditional research where it was not germane to his concerns" (p.281). To Reed's justification of this strategy — "Why crowd out the relevant for the sake of the irrelevant?" — the answer must surely be "Who is to judge what is relevant?". Elsewhere in the book, Reed defends Gibson's failure to mention Hubel and Wiesel's studies of the responses of visually driven cortical neurons on the grounds that they are "irrelevant" (p.138), but again he offers no justification for the claim. Finally, Reed also has the annoying tendency to treat Gibson as a figure as important in the development of scientific thought as Newton, Darwin and Einstein and to relegate all others to seeming irrelevance. For example, "Among all the great students of perception from the Greeks onward, Gibson was the *only* one who did not allow his commitment to scientific understanding to obscure the need to explain the meaningful aspects of perception" (p.183). Or "Nothing that was pertinent to a theory of perception was left out of Gibson's books" (p.138). Such claims only serve to alienate the sceptics.

Gibson's work deserves closer analysis as an alternative way of thinking about the nature and function of perception. The possibility of an ecological (computational) theory was the essence of his position and as such is clearly worthy of investigation. Computational theorizing offers the possibility of rigorously testing what are often dismissed as hand-waving ideas about the 'availability of information', while the largely sterile debate about the appropriateness of using the computer-based concept of 'representations' is best forgotten. Reed's book provides a detailed and scholarly account of the career of one of the most fascinating figures of twentieth-century psychology, and as such it deserves to be read. □

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Viral diversity

Stephen C. Inglis

RNA Genetics. Edited by Esteban Domingo, John J. Holland and Paul Ahlquist. CRC Press: 1988. Vol. I pp. 217, Vol. II pp. 249, Vol. III pp. 260. \$425. Distributed in Britain by Wolfe Medical £323.

MORE than 25 years ago, RNA was identified as an elusive messenger that transferred genetic information from DNA to protein. Since then it has emerged as a remarkably versatile molecule of complex structure and function, and is now widely regarded as the forerunner of DNA in the pre-biotic soup. The broad title of this three-volume series therefore leads one to expect a comprehensive treatment of this fascinating macromolecule. On closer inspection, however, it turns out to be an umbrella for a collection of 32 brief reviews which deal almost exclusively with RNA viruses. This could be regarded as perfectly fair, because only viruses use RNA as their genetic material. Nonetheless, a feeling of mild dissatisfaction is inevitable.

The broad theme at the heart of the series is a consideration of the genetic diversity of RNA viruses, how that diversity is generated, and what consequences it may have for virus evolution and disease. As underpinning, the first half of the contributions are devoted to describing the replication strategies for most of the recognized RNA virus groups. Viruses of bacteria, yeast, fungi and of course animals are represented, but there is particular emphasis on plants, with chapters on viroids and satellite viruses as well as more conventional infectious agents. The DNA-containing hepadnavirus and cauliflower mosaic virus groups are also covered along with the retroviruses, on the grounds that they replicate through an RNA intermediate, and these articles pave the way for Boeke's excellent review of the molecular biology of the closely related retrotransposons.

This is an impressive collection of chapters which ought to provide the teacher and advanced student, if not the expert, with a convenient and comprehensive source of information on basic mechanisms of replication; indeed the only serious omission I noted was of a section on the bunya and arenaviruses, whose unusual 'ambisense' genome arrangement should have ensured their inclusion. However, although most authors have opted, in the limited space available, for a broad overview of their subject (there is a particularly good contribution in this category from Coffin on retroviruses), others have produced much more specialized reviews. The opening chapter, for example, presents

a rather inaccessible mathematical treatment of the kinetics of Q β RNA replication, while another concentrates on the detailed characteristics of plant virus RNA-dependent RNA polymerases, which are of very limited interest.

The remaining chapters deal with genetic variability among the RNA viruses, an important subject in light of the notoriously mutable nature of RNA virus genomes. In most of the contributions the emphasis once again is on specific virus groups, and where genetic differences can be related to particular biological characteristics such as antigenicity or pathogenicity — as for the influenza and reoviruses — they are generally informative. More stimulating, however, are the chapters dealing with broader aspects of RNA virus heterogeneity. For example, there are good accounts of RNA recombination among the positive-strand viruses, and of the generation and possible biological significance of defective interfering viruses.

A consideration by Domingo and Holland of the error-prone nature of RNA virus replication provides a useful introduction to an exposition by Eigen and Biebrer of the 'quasispecies' theory of genome structure and its consequences for evolution and darwinian selection of RNA genomes. Finally, the evolution of RNA viruses is considered further in an interesting chapter by Zimmermann, who advances the notion of genetic 'modules' that may have been shuffled around between different RNA virus groups and may have given rise to unexpected genetic similarities.

The different approaches adopted by different authors impose a rather uneven quality on *RNA Genetics*, which will undoubtedly limit its appeal. There are chapters here to satisfy expert and student alike, and so these volumes would still represent a useful acquisition for the virologist's departmental library. But who's going to pay £300 for them? □

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New in paperback

● *Tilings and Patterns: An Introduction*, by Branko Grünbaum and G. C. Shephard, a corrected reprint of the first seven chapters of the authors' earlier book of 1987. Publisher is W. H. Freeman, price is \$26.95 (to be published in Britain in June, £18.95). The original volume (simply entitled *Tilings and Patterns*) was reviewed in *Nature* 326, 553 (1987).

● *Mindwaves: Thoughts on Intelligence, Identity and Consciousness* edited by Colin Blakemore and Susan Greenfield. Publisher is Basil Blackwell, price is £9.95, \$16.95. For review see *Nature* 330, 297 (1987).

● *Evolution: The History of an Idea*, by Peter J. Bowler, a revised edition that is also available in hardback. Publisher is University of California Press, price is pbk \$15.95, hbk \$48. For review see *Nature* 311, 587 (1984).

Changing places

Bernard Wood

A Theory of Human and Primate Evolution.
By Colin P. Groves. Oxford University
Press: 1989. Pp.375. £40, \$49.95.

COLIN GROVES is a delightfully anachronistic scientist. While most of us attempt to learn more and more about less and less, Groves's interests have broadened with time — the references of his that he cites in this book are an eclectic collection embracing work on marsupials, rhinoceroses and bovids.

In 1975 Groves surprised hominid palaeontologists with a novel and radical analysis of early *Homo* remains from East Africa. Its conclusions were awkward and, perhaps because of that, they have been largely ignored. But those who surmised that this was to be Groves's only incursion into hominid palaeontology are due for another surprise, for his new book shows that he must have been thinking about, and following, hominid evolutionary studies for the intervening 15 years. There is much of interest in the first five chapters, which deal with systematics, evolutionary theories and primate evolution. In this review, however, I will dwell on the sections of the book in which Groves sets forth his views about how the hominid fossil record should be interpreted.

The first of several adjustments the reader is invited to make is the author's adoption of the term 'hominin' in place of hominid. He proposes that the Family Hominidae embraces two subfamilies — Ponginae (which includes a single genus, *Pongo*), and the Homininae (embracing three tribes, the Gorillini, Panini and Hominini). The first two of these tribes are monogeneric; the last includes four genera, three of which are named (*Homo*, *Australopithecus* and *Paranthropus*), while the fourth is not.

Groves is a 'splitter' by inclination. Sometimes he subscribes to splits proposed by others, but in other cases his combinations are novel. Much of his detailed discussion centres on the systematics of the early hominid fossil record from African sites. The Hadar material is subdivided into 'small' and 'large' remains, the former (including the skeleton AL-288) being judged to be the "plesiomorphic sister group to all other Hominini", whereas the latter (including the numerous AL-333 remains) is allocated to an unnamed species of *Homo*. The Laetoli type fossil series is retained, as it must be, within *Australopithecus afarensis*, but its *Homo* affinities are emphasized. Groves is not prepared to subsume all the East African 'robust' australopithecines into a single species,

but few will have expected his preferred taxonomic solutions. *Paraustralopithecus aethiopicus* is transferred to *Homo*, the derived and primitive characters in KNM-WT 17000 are accorded separate specific status, and KNM-ER 732 and its ilk are placed in an unnamed species of *Paranthropus* whose similarity to *Paranthropus boisei* (for example crania KNM-ER 406 and OH5) is acknowledged. Groves follows Broom in recognizing two separate taxa of the 'robust' australopithecines from Southern Africa. In the light of recent suggestions by Clarke and others, it

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

New man? A frontal view of KNM-ER 1470, a 1.9-million-year-old *Homo* cranium from Koobi Fora, Kenya. It is generally thought to belong to *Homo habilis*, but Colin Groves regards it as being distinct from that taxon, and suggests that it should be placed in a new species, *Homo rudolfensis*.

is noteworthy that he sees no need for taxonomic rearrangement of the remains presently attributed to *Australopithecus africanus*.

As might be predicted, it is the treatment of *Homo* that is most radical. The allocations of material from Hadar to one taxon, and those from Omo Member C and the Upper Burgi Member at Koobi Fora to another, have already been alluded to. The author claims, and I believe him, that he sought, but did not find, evidence of heterogeneity in the Olduvai hypodigm of *Homo habilis*, so he adheres to his earlier conclusion that *H. habilis sensu stricto* remains a 'good' taxon. Several derived features are proposed to support this conclusion (p. 268), drawing upon evidence from the cranial vault and base and the dentition. Although the early *Homo* remains from Koobi Fora overlap in time with those attributed to *H. habilis* from Olduvai, the former are judged to show sufficiently consistent patterned heterogeneity to justify their allocation to several taxa.

Groves persists with *Homo ergaster*,

and by including KNM-ER 1813 within it he implies that this taxon spans nearly half-a-million years. Specimens such as KNM-ER 1470, 1590 and 1802 are included in a separate taxon *Homo rudolfensis*, which takes its species name from a suggestion made by Alexeev in 1986. The phenetic resemblances of this hypodigm to *H. habilis* are recognized, but the latter group is kept separate because of its possession of derived traits which are shared with later *Homo* taxa. Groves claims that *H. rudolfensis* can be distinguished by the unique combination of relative canine size and mandibular symphyseal form. The crania KNM-ER 3733 and 3883 are also recognized as a separate species, said to share derived traits with *H. ergaster*, but which is nonetheless judged to be distinct from *Homo erectus*.

What should we make of all this? Can these taxonomic assessments be dismissed as the byzantine ramblings of a zoologist who is unacquainted, at first hand, with much of the fossil evidence? Such a judgement would be both unfair and unwise. Groves's wide experience with other animal groups is just the background that many hominid palaeontologists lack and I, for one, will give his judgements careful consideration. We must also remember that taxonomic solutions as complex as these have been supported by other experienced zoologists, Ian Tattersall for example. This is not to say that Groves's solutions cannot be faulted. Several of them can; for example, I believe that the affinities of KNM-ER 992, the type specimen of *H. ergaster*, have, once again, been misjudged.

This is an unconventional and stimulating book. It gives welcome emphasis to geographical variation and helps blow the dust off the conventional wisdom of hominid palaeontology. Groves includes in the text a cladistic analysis of hominids which once again demonstrates the apparently high levels of convergence which bedevil the efforts of hominid palaeontologists to determine durable phylogenetic schemes. It also delivers, as the title promises, a new theory for primate and hominid evolution based on the neglected precepts of Berg.

Aspects of the book's production deserve criticism. Groves needs to buy a scale, a new camera or a new pair of spectacles; I suspect he needs all three. And the publishers should select their proofreaders a good deal more carefully; the text is blemished by too many typographical and citation errors. But this refreshing and original volume should not be ignored. John Napier, one of the two people to whom it is dedicated, would have enjoyed reading it. □

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National Museums of Kenya

The bright side

Desmond King-Hele

Is Science Necessary? Essays on Science & Scientists. By Max F. Perutz. *E.P. Dutton, New York: 1989. Pp.285. \$19.95. To be published in Britain on 13 July by Barrie & Jenkins, £14.95.*

"Is scientific research the noblest pursuit of the human mind, from which springs a never-ceasing stream of beneficial discoveries, or is it a sorcerer's broom that threatens us all with destruction?" The stark alternatives set out in the opening sentence of this book nicely spotlight its theme. Max Perutz, who in 1962 shared the Nobel prize with John Kendrew for their work on haemoglobin and myoglobin, has enjoyed a splendid career buoyed by faith in the first alternative. Now he is dismayed and puzzled by the anti-scientific campaigns that make such a noise all round the world, often propagated by pressure-groups ignorant of science.

In the first half of the book he takes a cool scientific look at some of the problems facing the world, especially in food production, health and sources of energy. The second half, devoted to wartime reminiscences and essay-reviews of various books on recent scientists or advances in science, shows the human side of science and the passionate intensity of its practitioners. A chain of essays is a dangerous formula for a book: it throws a great strain on the quality of the writing, because one weak link can destroy the will to read. I am happy to say that I found this collection enthralling, right to the end, though the book's title may make scientists wince: presumably it was chosen as a bait for the anti-science activists who would greatly benefit from reading it.

Most scientists will agree with most of the conclusions in Perutz's survey of world food, health and energy. Not everyone will agree with everything however, because we all have unconscious or semi-conscious biases and he is no exception. On the subject of food he tries hard to be rational and balanced, and largely succeeds. He rightly emphasizes the benefits science has brought, through high-yield crops resistant to disease. What is a little lacking is the ecological dimension, and there is an air of complacency about the long-term effects of fertilizers and pesticides. On health, he brings out well the dilemma that all benefits carry some risk, and the consequent problem, that trying to eliminate all risk may stifle future advances. There are many good diagrams showing the benefits of drugs and vaccines, but one, Fig. 14, giving deaths from tuberculosis, is rather bogus because the decrease in deaths during the pre-antibiotic era is extrapolated linearly on a logarithmic

scale, thus ensuring it can never reach zero. (Replotting on a linear scale quite alters the conclusions to be drawn.)

The discussion of energy policy is generally excellent, though wave and geothermal power are not mentioned and not everyone will be happy to find Perutz championing nuclear power. If he wanted to convince sceptics, he should not have accepted so blithely the estimates of accident probabilities of "less than 1 in 10 million reactor years": such low probability estimates are worthless, and leave unanswered those who ask, "what about a crashing jumbo-jet or a hostile missile in the next 100 years?". It would be unfair to make too much of these small blemishes in a survey that is scientific, humane and appealing.

In contrast the next essay, "Enemy Alien", is inhumane and appalling. In May 1940, four years after arriving in Britain as a refugee from Austria, and

having recently gained his PhD, Perutz was rounded up as an 'enemy alien' and thrown into prison before being deported to Canada with 1,200 others in a troopship. He fared better than the previous cargo of 1,400 aliens, of whom 600 drowned when their ship was torpedoed, but the months spent in prison camps in Canada left their mark. Although Perutz never says so, I suspect that for him and for other refugee-scientists imprisoned with him, including Hermann Bondi and Thomas Gold, this experience in prison has made the rest of life seem rather easy. Perhaps that is why, even in the Britain of today, they are so resilient and optimistic, convinced that culture and science will somehow prevail. Be that as it may, Perutz's book is a service to science and a pleasure to read. □

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Togetherness

John Barrett

Coevolution of Fungi with Plants and Animals. Edited by K. A. Pirozynski and D. L. Hawksworth. *Academic:1988. Pp.285. £35, \$68.*

It is difficult to know what to make of this collection of essays. The editors set out with the commendable objective of bringing examples of the many and varied interactions between fungi and other organisms to the attention of evolutionary biologists. But because the contributions have been written, or edited, with such a strict adherence to the proprieties of fungal terminology and taxonomy, they are likely to be tough going for non-mycologists.

As Pirozynski and Hawksworth point out in their long introductory chapter, the fact that most fungi are obligate, and often mutualistic, symbionts has led most mycologists to take coevolution for granted. In consequence, research on fungal symbioses has tended to lag behind that in other fields. The editors also confess that the number of examples that offer direct evidence of coevolutionary processes at work is very limited; they have, therefore, given the contributors a licence for 'just-so' story-telling, because without such licence "this book might not have materialised" (p. 4).

The result is a rather mixed bag of examples, ranging from instances in which there is some experimental evidence, through those with no empirical support but with testable hypotheses, all the way to those which, not to put too fine a point on it, verge on fantasy. Fortunately, most of the authors present their topics agnos-

tically. For example, Evans introduces his entertaining review of entomogenous fungi with the disclaimer that "The facts are scarce and the evidence when available, is circumstantial and thus controversial. I therefore make my apologies in advance".

Coevolution can be studied at many levels. The contributions reflect this, running from studies of the 'congruence' of phylogenies of plant species and their fungal parasites (Hijwegen) down to studies at the molecular level (Pirozynski).

The value of the book is that the areas of our ignorance are laid bare. An excellent example is the case of ambrosia galls, which are formed by an interaction between a gall-forming midge, its plant host and a fungus which infects the gall and provides food for the developing midge larva. Here, one would have thought, there would be a clear *prima facie* case for coevolution. Yet, as one reads through Bissett and Borkent's thorough review, one begins to have doubts. Eventually these doubts are confirmed by the authors' own conclusions. Other cases of symbiotic interactions that arose through coevolution are, however, more convincing. In his excellent account of fungal endophytes of grasses, Clay presents good *experimental* evidence to justify his argument that coevolutionary processes have here produced a shift from parasitism to mutualism.

There is much in this volume to interest any student of coevolution. Although the mycological jargon is intimidating for the non-specialist, wading through it is probably worth the effort and will provide a rewarding insight into the evolution of fungal symbioses. □

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Structural plasticity broadens the specificity of an engineered protease

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The substrate specificity of α -lytic protease has been changed dramatically, with a concomitant increase in activity, by replacing an active-site Met with Ala. The substrate specificity of both this mutant and another similar mutant are extraordinarily broad. X-ray crystallographic analysis shows that structural plasticity, a combination of alternate side-chain conformations and binding-site flexibility, allows both large and small substrates to be well accommodated.

ONE of the fundamental functions of an enzyme is to provide specificity by limiting the range of substrates which are catalytically productive. As catalysis is a result of an enzyme's ability to bind the transition state for a reaction, substrate specificity derives from the selective binding of transition states for the reactions of favoured substrates¹. Although specificity must result from the complex interplay between protein and substrate of hydrogen bonding, van der Waal's and electrostatic forces, steric repulsion and the hydrophobic effect, the way in which these factors are combined to achieve particular patterns of substrate specificity remains unclear.

One of the simplest questions that can be addressed is whether the specificity of an enzyme can be altered without destroying catalytic activity. Recently, there has been some success in modifying the substrate specificity of subtilisin²⁻³ and lactate dehydrogenase⁴. Understanding the structural basis for these successes, as well as other failures^{5,6}, however, is limited. In this work we report that the pattern of substrate specificity of α -lytic protease can be changed dramatically by replacing an

active site methionine with alanine. The high level of activity and exceptionally broad pattern of substrate specificity observed for this mutant are remarkable. Another similar mutant displays even less specificity, but at a reduced level of activity. An extensive X-ray crystallographic analysis, which focuses on complexes of the protease with substrate analogues, suggests that the altered specificity of these mutants results from structural flexibility at the enzyme active site.

α -Lytic protease

α -Lytic protease, an extracellular bacterial serine protease and structural homologue of the mammalian enzymes, catalyses peptide-bond hydrolysis by stabilizing a high-energy, oxy-anionic, tetrahedral intermediate formed by the nucleophilic attack of Ser 195 (numbered from 15-244(A) by alignment with chymotrypsin⁷) on the carbonyl of the scissile bond⁷⁻¹². The enzyme is specific for Ala in the primary specificity or P₁ position (the residue before the scissile bond, with residues P₂, P₃ and so on, extending towards the N-terminus¹³) of peptide-*p*-Na substrates with the structure sAAP-X-*p*-Na, where sAAP is succinyl-Ala-Ala-Pro, X is Ala, Val, Met, Leu or Phe, and *p*-Na is *para*-nitroanilide (ref. 14; Fig. 1). On these and related peptide substrates¹⁵, values of k_{cat}/K_M fall off dramatically as the size of the residue at this position increases (Fig. 1). The value k_{cat}/K_M (catalytic efficiency) is the apparent second-order rate constant for the reaction of enzyme and substrate to form product; the log (k_{cat}/K_M) is proportional to the transition-state binding energy of the enzyme and substrate for simple reactions such as peptide hydrolysis¹.

Remodelling substrate specificity

Mutations designed to change the specificity of the enzyme were chosen on the basis of the structures of complexes between the

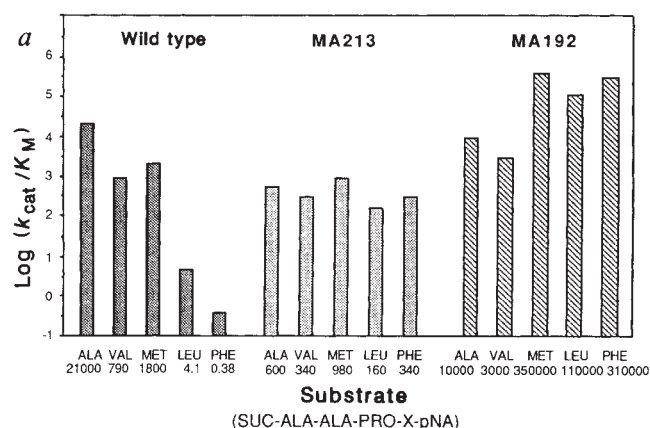


FIG. 1 *a*, The effect of mutations on specificity. Log(k_{cat}/K_M) is shown as a bar and numerically for each of five substrates, sAAP-X-*p*-Na where X = Ala, Val, Met, Leu, and Phe, for the wild-type enzyme and mutants Met $\rightarrow$ Ala 192 (MA192), and Met $\rightarrow$ Ala 213 (MA213). *b*, Tabulated values of k_{cat} and K_M for each enzyme.

METHODS. The Met $\rightarrow$ Ala 213 mutation was constructed by site-specific mutagenesis²², using the oligonucleotide 5'TGCCGCCGACGCCAGCCCTGC3'. Met $\rightarrow$ Ala 192 was constructed by cassette mutagenesis²³ using the complementary oligonucleotides

Substrate	Wild Type		MA213		MA192	
	k_{cat} (s ⁻¹)	K_M (mM)	k_{cat} (s ⁻¹)	K_M (mM)	k_{cat} (s ⁻¹)	K_M (mM)
sAAP-Ala- <i>p</i> -Na	75 $\pm$ 9	3.6 $\pm$ 0.4	34 $\pm$ 7	57 $\pm$ 11	37 $\pm$ 5	3.6 $\pm$ 0.05
sAAP-Val- <i>p</i> -Na	13 $\pm$ 0.2	16 $\pm$ 0.3	10 $\pm$ 3	29 $\pm$ 10	3.4 $\pm$ 0.01	1.1 $\pm$ 0.003
sAAP-Met- <i>p</i> -Na	56 $\pm$ 6	31 $\pm$ 3	---	---	120 $\pm$ 4	0.33 $\pm$ 0.01
sAAP-Leu- <i>p</i> -Na	1.2 $\pm$ 0.9	290 $\pm$ 230	---	---	87 $\pm$ 3	0.77 $\pm$ 0.024
sAAP-Phe- <i>p</i> -Na	0.0068 $\pm$ 0.0007	17 $\pm$ 2	47 $\pm$ 8	16 $\pm$ 3	130 $\pm$ 3	0.40 $\pm$ 0.008

5'GGCCAGCGCA3' and 5'CGCGTGGCTGGCCGC3' inserted into pALP5²². α -Lytic protease and mutants were purified^{24,25} from culture filtrates of *Escherichia coli* expressing the protease gene²². Values of k_{cat}/K_M were determined from the slopes of double reciprocal plots of initial reaction velocity as a function of substrate concentration and values of k_{cat} and K_M from the intercepts. Enzyme concentrations were determined from the absorbance of stock solutions at 280 nm²⁴. Substrate inhibition was observed for the hydrolysis of sAAP-Leu-*p*-Na by the wild-type enzyme and for the hydrolysis of sAAP-Met-*p*-Na and sAAP-Leu-*p*-Na by Met $\rightarrow$ Ala 213. For these cases, values of kinetic parameters were extrapolated from measurements at low substrate concentration. The estimated errors for values of k_{cat}/K_M averaged 2.2% and ranged from 1% to 5%.

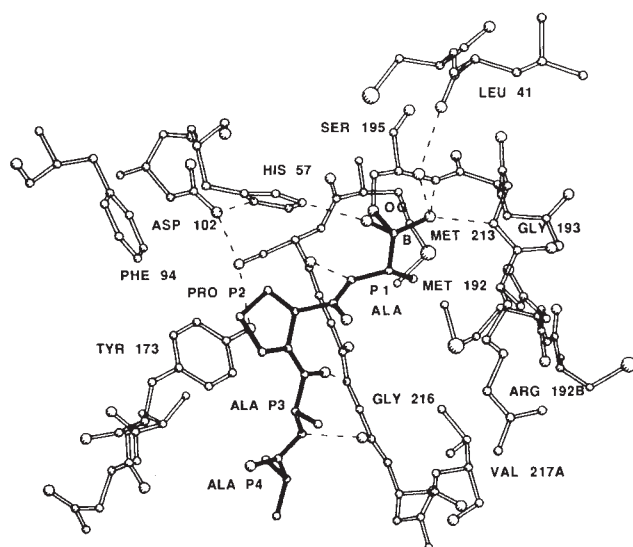


FIG. 2 The structure of the complex formed between the enzyme and mAAP-boroAla ($K_i = 67$ nM; refs 14 and 16). Note the seven hydrogen bonds (dotted lines) that stabilize the tetrahedral adduct.

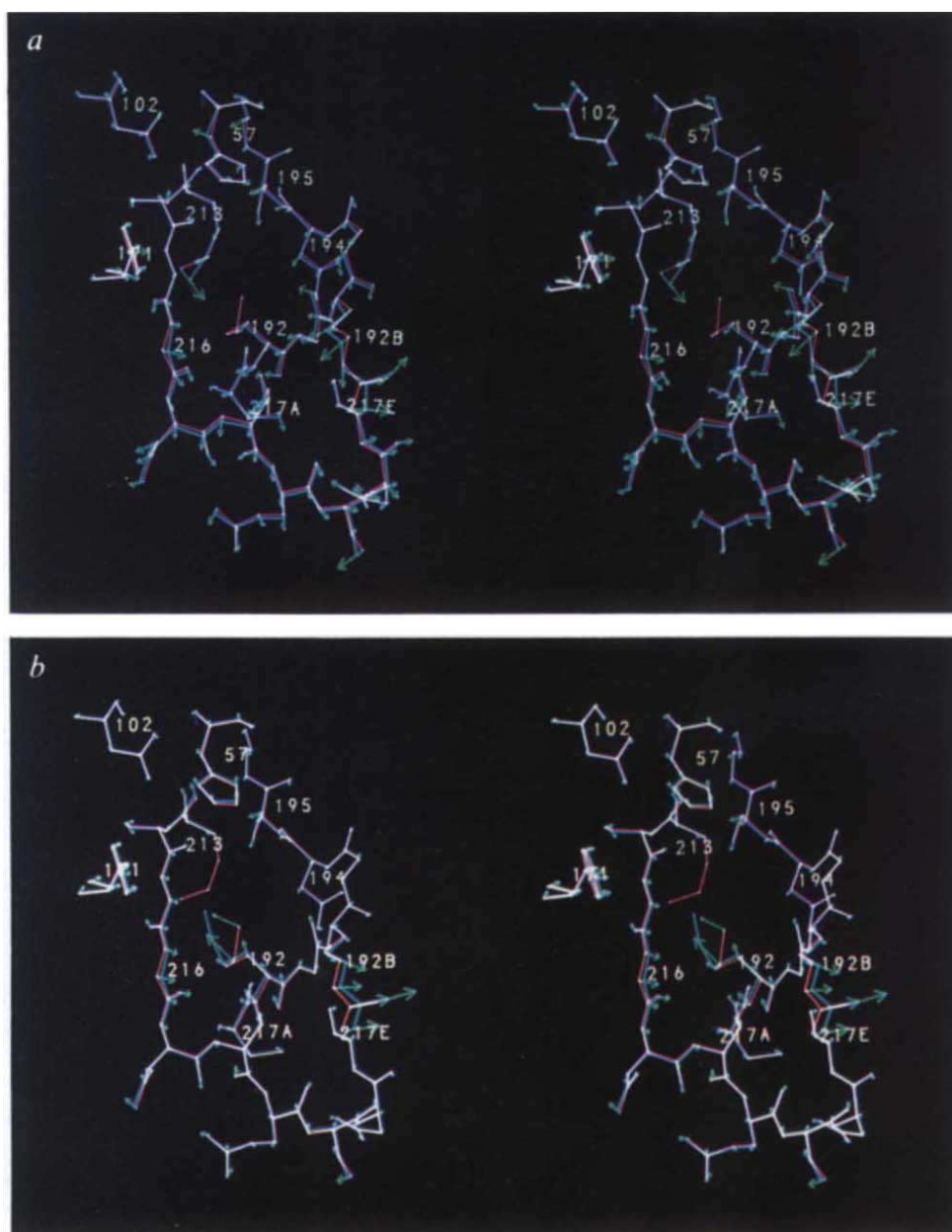


FIG. 3 Stereo drawing of the active-site region of mutant α -lytic proteases (A = Met $\rightarrow$ Ala 192, B = Met $\rightarrow$ Ala 213) shown in blue are superimposed on the active site region of the wild-type enzyme^{7,8} shown in red. The slight collapse of the binding pocket in the mutants are indicated by arrows representing the interatomic distance vectors, multiplied fourfold, between the atoms of wild-type and mutant enzymes. Coordinate sets were aligned by minimizing the deviation between all respective C_α atoms. For Met $\rightarrow$ Ala 192 (a) the removal of Met 192 from the specificity site causes the top and bottom of the specificity pocket and the residues surrounding it to move inwards, whereas the side of the pocket containing Val 217A moves away from the centre of the pocket. In Met $\rightarrow$ Ala 213 (b), Met 192 occupies two conformations in this structure: one in which C_ϵ points back into the pocket towards Trp 199 with 20% occupancy, and the other in which C_ϵ points in the reverse direction out of the binding site with 80% occupancy. In the latter, a water slips in behind Met 192. The lowest occupancy conformation of Met 192 is shown. Mutation of Met 213 to Ala seems to cause small movements that push in on the bottom of the specificity pocket (residues 214–216), as in Met $\rightarrow$ Ala 192. The top of the pocket (residues 192–193) moves towards Ala 213, rather than bowing into the binding site, which is consistent with the rearrangement of Met 192.

enzyme and inhibitory peptide boronic acids (Fig. 2; refs 12 and 14). These peptide analogues, in which the C-terminal carboxyl group has been replaced with a boronic acid $(\text{B}(\text{OH})_2)$, form covalent tetrahedral adducts with the active site Ser. Such complexes are excellent mimics of the high-energy tetrahedral intermediates formed during substrate hydrolysis^{12,14,16} and hence the enzyme-substrate transition state^{12,14}. It seemed that specificity for Ala in the P_1 position was conferred by the presence of the side-chain of Met 192, which occluded what would otherwise be a deep binding pocket (Fig. 2). This pocket is closed on the top by residues 192–193, on the bottom by residues 214–216, and on the sides by the side-chains of residues Met 213 and Val 217A. Conversion of Met 192 to Ala (Met $\rightarrow$ Ala 192) would be expected to create a pocket just large enough to accommodate a Met residue in the P_1 position of the substrate, but too small for larger or branched side-chains. Converting Met 213 to Ala (Met $\rightarrow$ Ala 213) would increase the volume of the pocket by the same increment, but provide a different geometry.

To establish how the substrate specificity of the mutants had changed, values of k_{cat} , K_M and k_{cat}/K_M were again determined

for each of the mutant proteases with each of the five substrates (Fig. 1). For the Met $\rightarrow$ Ala 192 mutation, the maximal activity of α -lytic protease shifted from Ala to Met in the P_1 position of the substrate. Catalytic efficiency for sAAP-Met-*p*-Na increased 200-fold relative to the wild-type enzyme, to a level that was 17 times greater than the value for the wild-type enzyme with the best wild-type substrate. Even more dramatic was the nearly six orders of magnitude change in catalytic efficiency of the mutant towards sAAP-Phe-*p*-Na ($(k_{\text{cat}}/K_M \text{ Met} \rightarrow \text{Ala 192})/(k_{\text{cat}}/K_M \text{ wild-type}) = 840,000$) and over four orders of magnitude for sAAP-Leu-*p*-Na ($(k_{\text{cat}}/K_M \text{ Met} \rightarrow \text{Ala 192})/(k_{\text{cat}}/K_M \text{ wild-type}) = 26,000$). These changes in specificity result from the removal of the equivalent of just three methylenes ($80 \text{ \AA}^3$) from the specificity pocket of the enzyme. Improved catalytic efficiency is primarily the result of increased affinity of the mutant enzyme for the Michaelis complex of good substrates, with only small increases in k_{cat} (Fig. 1*b*).

Surprisingly, enlargement of the binding pocket, which resulted in such a dramatic improvement in the catalytic efficiency of the Met $\rightarrow$ Ala 192 mutant towards sAAP-Phe-*p*-Na, only decreased activity towards the corresponding Ala substrate

FIG. 4 Stereo diagram of the superimposed structures of the complex formed between Met $\rightarrow$ Ala 192 and mAAP-boroPhe (blue) and the complex formed between the wild-type enzyme and mAAP-boroAla (red). The K_i value for mAAP-boroPhe was calculated from the rate constants for association ($k_{\text{on}} = (2.0 \pm 0.08) \times 10^5 \text{ s}^{-1}$) and dissociation ($k_{\text{off}} = (1.16 \pm 0.23) \times 10^{-4} \text{ s}^{-1}$) of the complex, which were determined as described by Williams and Morrison²⁶. Polylysine at 0.1 mg ml^{-1} was used to stabilize the enzyme at very low concentrations. The r.m.s. deviation in the C_α coordinates of the mutant inhibitor complex relative to the unliganded mutant is $0.17 \text{ \AA}$.

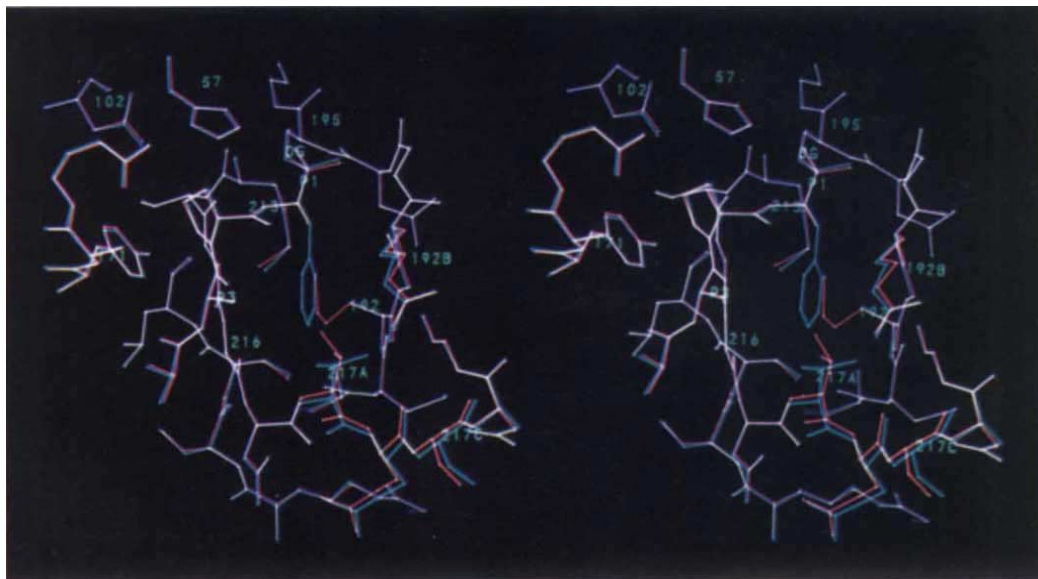
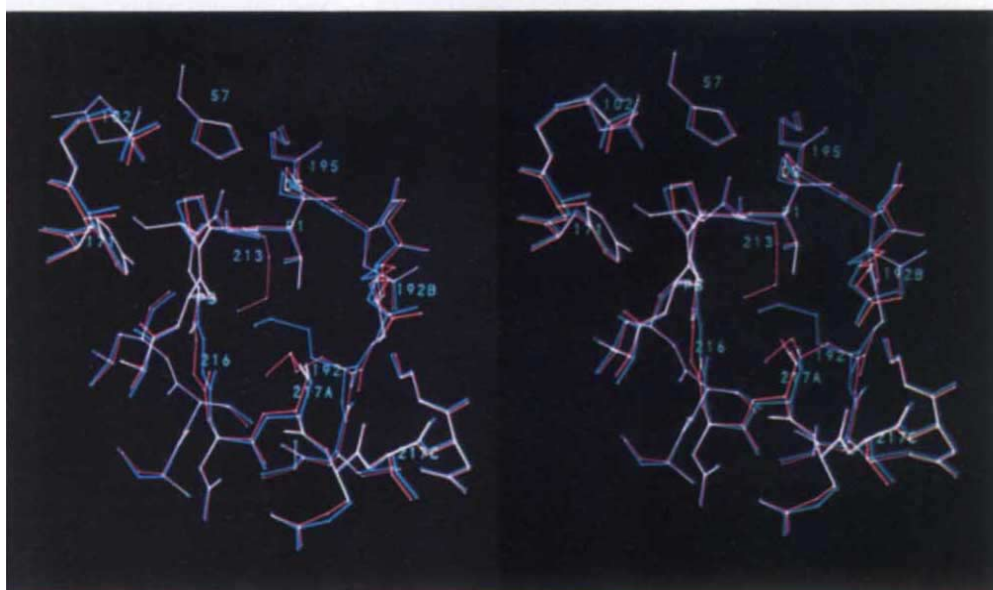


FIG. 5 Stereo diagram of the superimposed structures of the complex formed between Met $\rightarrow$ Ala 213 and mAAP-boroVal (blue) and the complex formed between the wild-type enzyme and mAAP-boroVal (red; ref. 14). The dissociation constant for the binding of mAAP-boroVal to Met $\rightarrow$ Ala 213 was determined by monitoring reaction velocity as a function of inhibitor concentration at substrate concentrations far below the K_M ($[S] = K_M/20$). Under these conditions the K_i value is the reciprocal slope of a plot of the ratio of initial reaction velocity in the absence of inhibitor to the initial reaction velocity in the presence of inhibitor as a function of inhibitor concentration. The r.m.s. deviation in the C_α coordinates of the mutant inhibitor complex relative to the unliganded mutant is $0.11 \text{ \AA}$.



twofold. The ability of the Met→Ala 192 mutant enzyme to distinguish between Ala and Phe substrates ($((k_{\text{cat}}/K_{\text{M Phe}})/k_{\text{cat}}/K_{\text{M Ala}})=32$) is much lower than that of either the wild-type enzyme ($((k_{\text{cat}}/K_{\text{M Ala}})/(k_{\text{cat}}/K_{\text{M Phe}})=55,000$) or chymotrypsin ($((k_{\text{cat}}/K_{\text{M Phe}})/(k_{\text{cat}}/K_{\text{M Ala}})=50,000$ for methyl ester substrates¹⁷), a serine protease with specificity for large hydrophobic P₁ substrates^{17–19}. The mutation therefore makes α -lytic protease a highly active enzyme with exceptionally broad substrate specificity.

Whereas mutation of Met 192 to Ala significantly decreases the selectivity of the enzyme, mutation of Met 213 to Ala completely eliminates the selectivity (Fig. 1). The best substrate for the Met→Ala 213 mutant, sAAP-Met-*p*-Na, is hydrolysed only six times more rapidly than the worst substrate tested, sAAP-Leu-*p*-Na. These results illustrate the complexity of enzyme–substrate interactions, in that both mutations increase the volume of the specificity site by the same amount but have quite different functional effects.

For both mutants, the dependence of k_{cat} , K_{M} , or $k_{\text{cat}}/K_{\text{M}}$ on the substrate side-chain does not seem to be correlated with properties of the substrate, such as side-chain volume or side-chain hydrophobicity. This is in marked contrast to subtilisin³ and chymotrypsin^{17–19}, whose specificities can be understood simply in terms of the partitioning of the non-polar substrate side-chains into a hydrophobic binding pocket. Factors other than binding-site volume, such as residue packing, conformational strain, or conformational changes must be important in determining the specificity of the mutant enzymes.

New specificity sites

Analysis of the structure of unliganded Met→Ala 192 (Table 1, Fig. 3a) shows that replacement of Met 192 with Ala creates a deep hole in the specificity pocket that is partially filled by a solvent molecule, but results in no major movement in the residues surrounding the pocket. As the pocket remains intact, we assume that the enthalpic and entropic price of maintaining this hydrophobic hole in the specificity pocket has been paid for by protein–protein interactions that maintain the folded state.

When Met 213 is converted to Ala, the specificity pocket is enlarged by the same volume as in the Met→Ala 192 mutant. However, Met 192 adopts a new conformation in which its side-chain moves towards the position occupied by Met 213 in

the wild-type enzyme (Fig. 3b). The C_ε of Met 192 is disordered, occupying two conformations separated by a 120° rotation about the C_γ–S_δ bond. In one of the conformations, a water is bound behind Met 192 and forms a hydrogen bond to the ring nitrogen of Trp 199, to the carbonyl of residue 203 and possibly to the sulphur of Met 192. The driving force behind this conformational change may be that the new position places the side-chain of Met 192 in a more hydrophobic environment than it normally occupies in the wild-type enzyme, where it is packed over a polar Ser–Gln hydrogen-bonded pair. No rearrangement of Met 213 occurs in the Met→Ala 192 mutant, presumably because the side-chain is already in the most nonpolar environment available. In Met→Ala 213 the P₁ binding site is broader than the wild-type binding site, but remains shallow, allowing it to accommodate large side-chains. The considerably smaller Ala side-chain, however, would not be expected to interact as well with the altered binding site.

Although the structural changes in both unliganded mutants are quite small, they seem to be the result of concerted movements of large sections of the protein that occur as a consequence of the mutation (Fig. 3a, b). These small adjustments in the atomic positions (0.1–0.4 Å) are non-random and show consistent patterns of motion over long distances, and so do not seem to derive from errors in the structure at this resolution (2.15 Å) or to be artefacts of refinement. Thus, creating a hole in a hydrophobic region of the protein leads to a decrease in the width of the specificity site by up to 0.4 Å.

Substrates induce conformational changes

The structure of the complex formed between the Met→Ala 192 mutant and mAAP-boroPhe (Table 1), where mAAP is methoxysuccinyl-Ala-Ala-Pro and 'boro' indicates that the Phe residue is the α -aminoboronic acid analogue of Phe, reveals why the enzyme is such a good catalyst for the hydrolysis of sAAP-Phe-*p*-Na (Fig. 4). In parallel with changes observed in catalytic efficiency, mAAP-boroPhe is bound by the mutant enzyme 100-fold more tightly ($K_i=0.58\pm0.12$ nM) than the wild-type enzyme binds the homologous Ala inhibitor, mAAP-boroAla ($K_i=67$ nM, ref. 16). As with peptide boronic acid binding to the wild-type enzyme¹⁴, mAAP-boroPhe forms a covalent tetrahedral adduct with the active-site serine that is stabilized by seven hydrogen bonds with the enzyme. These structures are essentially identical, except that in the Met→Ala 192 mutant the side-chain of Val 217A rotates 120° about the C_α–C_β bond to remove the methyl group, C_{γ1}, from the specificity pocket (Fig. 4). In addition, Val 217A moves 0.5–0.8 Å away from the binding pocket, as do residues 217B–217D to a lesser extent. The overall effect of these movements is to enlarge the pocket (by 2 Å in one dimension) sufficiently to accommodate the Phe side-chain of the inhibitor. The Phe ring is almost completely buried in the specificity pocket, resulting in a 25% improvement in the total hydrophobic surface buried on complex formation compared with the wild-type complex (calculated according to refs 20 and 21). This increase in buried surface area partially accounts for the improvement in the affinity of the mutant for the Phe-containing inhibitor as compared with the wild-type complex.

The observation that the conformation of the Met→Ala 192 mutant is altered upon binding the Phe-inhibitor implies that similar alterations occur during the hydrolysis of Phe-substrates. Because the conformational change is not observed in the free mutant enzyme, we reason that for substrates having large P₁ side-chains some of the enzyme–substrate association energy is being used to alter the enzyme conformation rather than to stabilize the transition state. If substrates with P₁ side-chains smaller than Phe do not require the conformational changes that allow mAAP-boroPhe to be accommodated by the enzyme, then their full interaction energy may be used during catalysis. This differential use of binding energy provides an explanation for why catalytic efficiencies for the series of hydrophobic

TABLE 1 Crystallographic parameters of α -lytic protease mutants

Structure	Resolution (Å)	[Inhibitor] (mM)	% Reflections I > 3 σ	R-factor	r.m.s. deviation (Å)
Met→Ala 192	2.15	—	80	0.151	0.16*
Met→Ala 213	2.20	—	89	0.135	0.09*
Met→Ala 192+ mAAP-boroPhe	2.25	100	86	0.139	0.09†
Met→Ala 213+ mAAP-boroVal	2.25	40	81	0.144	0.11†

Crystals of mutant enzymes, isomorphous with native crystals, were grown from 1.3 M Li₂SO₄ containing 20 mM Tris-sulphate by the vapour diffusion method using sub-microscopic native crystals as seeds^{8,12}. Enzyme–inhibitor complexes were prepared by adding an aliquot of aqueous inhibitor solution (1 μ l of either 0.1 M mAAP-boroPhe for (5 μ l)) containing 1–3 crystals of mutant protein and equilibrating overnight. Data for unliganded Met→Ala 213, Met→Ala 192 complexed with mAAP-boroPhe and Met→Ala 213 complexed with mAAP-boroVal were each collected from two crystals using a diffractometer and monochromatic copper K_α radiation. Data for unliganded Met→Ala 192 were collected from a single crystal using mirror-focused copper K_α radiation and a multiwire area detector. Structural changes and inhibitor locations were determined from difference Fourier maps using native α -lytic protease coordinates to generate calculated structure factors and phases and the new coordinates were refined by the stereochemically restrained least-squares algorithm of Hendrickson and Konneret^{12,27} using standard weights for the restraints, resulting in r.m.s. deviations from ideality of 0.016–0.022 Å for bond lengths and 3.3°–4.0° for bond angles.

* r.m.s. deviation in C_α coordinates relative to wild-type enzyme⁷.

† r.m.s. deviation in C_α coordinates relative to complex between wild-type enzyme and mAAP-boroAla.

substrates do not correlate with either P_1 side-chain hydrophobicity or size.

A similar structural analysis was carried out for the Met→Ala 213 mutant. The mAAP-boroVal (Fig. 5, Table 1) complex with the mutant is a factor of 30 less stable ($K_i = 200 \pm 7$ nM) than the complex formed between the wild-type enzyme and this inhibitor ($K_i = 6.4$ nM; ref. 16). As in the wild-type enzyme¹⁴, the tetrahedral adduct formed between the active site Ser and the boron of the inhibitor is stabilized by seven hydrogen bonds. In the mutant complex, however, the side-chain of Met 192 lines the very back of the specificity pocket, placing C ϵ in almost the same position that C ϵ of Met 213 occupies in the wild-type enzyme. Residues lining the pocket move inwards to optimize packing around the Val side-chain (Fig. 5). Although the solvent-accessible surface area buried on complex formation is the same in the mutant and wild-type complexes, the fit of the Val side-chain of the inhibitor into the specificity pocket is much poorer and leaves three gaps, each only slightly smaller in size than the volume of a methylene group. Consistent with poorer complementarity, the inhibitor exhibits considerably more thermal motion in the mutant complex relative to the wild-type enzyme, as indicated by a change in temperature factors for the entire inhibitor from an average value of $10 \text{ \AA}^2$ for the wild-type complex¹⁴ to $20 \text{ \AA}^2$ for the mutant complex.

In addition to the observed lack of complementarity, the stability of the complex might also be reduced by the shift of Gly 216 towards Met 192 which lengthens by $0.2 \text{ \AA}$, and presumably weakens, two hydrogen bonds between Gly 216 and the P_3 Ala residue of the inhibitor. The shifted position of Gly 216 in the mutant complex seems to be a result of the rearrangement of Met 192, which packs against Gly 216 in the wild-type complex. Taken together, these differences are probably sufficient to explain the 30-fold decrease in affinity of the mutant for the inhibitor.

Mutation of Met 213 to Ala has produced an enzyme with even broader specificity than the Met→Ala 192 mutant, but with lower catalytic efficiency. Exceptionally broad specificity seems to derive from the ability of the remaining residues in the specificity pocket, Met 192 and Val 217A (by analogy to the Met→Ala 192-mAAP-boroPhe complex), to adopt alternate side-chain conformations and from the main-chain flexibility of residues 215–216 and 217A–217C. The possibility that a solvent molecule is buried in the specificity pocket when inhibitors or substrates with an Ala-side-chain in the P_1 position interact with the enzyme, as in the structure of unliganded Met→Ala 213 mutant, may be responsible for decreased activity towards sAAP-Ala-*p*-Na. For substrates with larger P_1 residues, the presence of Met 192 lining the back of the specificity site may prevent efficient packing of the P_1 side-chain in the pocket, or force the side-chain out of the pocket enough to distort the hydrogen bonds stabilizing the enzyme–substrate complex.

Value of structural analysis

The premise of this work, based on computer graphics analyses of the liganded and unliganded wild-type enzyme, was that substitution of Ala for Met 192 of α -lytic protease could be complemented by substitution of Met for Ala in the P_1 position of substrates. Indeed, sAAP-Met-*p*-Na proved to be an excellent substrate for the mutant, with k_{cat}/K_M improving 200-fold to a level 17-fold above the best native enzyme–substrate combination. Surprisingly, sAAP-Phe-*p*-Na also functioned extraordinarily well as a substrate, with k_{cat} and K_M equivalent to the Met substrate, despite indications from the computer graphics analysis that the Phe side-chain was too large to fit into the specificity pocket. In addition, k_{cat}/K_M for sAAP-Ala-*p*-Na only decreased twofold, although contacts in the binding site were presumably lost.

Our results indicate that to understand the structural basis for the effects of mutations on the functioning of an enzyme, the interactions between the enzyme and substrate must be analysed in addition to the structure of the mutant enzyme alone. We also show that the results of even conservative mutations may be complicated by structural rearrangements induced on complex formation.

The structural basis of specificity

Structural studies of the wild-type enzyme and enzyme–inhibitor complexes indicated that the free enzyme was a rigid molecule, preformed to be complementary to the transition state. A shallow, nonpolar specificity site selects for small hydrophobic substrate side-chains and excludes large side-chains. In this work we show that the basic structure of the specificity pocket can also accommodate large side-chains if mutations are introduced in the binding site that increase pocket volume. The surprising kinetic behaviour of the mutant proteases is not consistent with simple models derived from chymotrypsin¹⁷ and subtilisin³, in which the specificity site behaves as a large, static hydrophobic pocket.

It seems that the exceptionally broad specificity is a consequence of structural plasticity in the mutant proteases. That the mutation does not simply increase the amount of binding site disorder is evidenced by the observation that the temperature factors for the active-site region do not change significantly upon mutation. The combined ability of residues 217A–217D to shift away from the binding pocket and for the side-chain of Val 217A to rotate out of the binding site allows large side-chains to fit into the specificity pocket. In addition, the flexibility of the main-chain atoms forming the top and bottom of the pocket allows them to bend into the site and interact more favourably with small substrate side-chains and to bow outwards for large side-chains. Introduction of these characteristics into enzyme active sites may be a general way of engineering broad rather than precise substrate specificity. Conversely, increasing the rigidity of a binding site should lead to more precise specificity. □

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Silicon carbide and the origin of interstellar carbon grains

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THE mechanisms of interstellar grain formation are not well understood. Such grains are complex^{1,2}, with components that include silicates, ices and carbonaceous material. Several forms have been proposed for interstellar carbon, including graphite³, amorphous carbon⁴, polycyclic aromatic hydrocarbons (PAHs)⁵, diamond⁶, fullerenes⁷ and bacteria⁸. The stellar winds of carbon-rich red-giant stars are the dominant source of interstellar carbon grains^{9–11}. However, the processes leading to the formation of grains are usually discussed only in the general terms of thermodynamics and classical nucleation theory^{12,13}. One possibility⁹ is that PAH condensation initiates the formation of grains, similar to the formation of soot in hydrocarbon pyrolysis and combustion. Our recent computational study¹⁴ demonstrates by detailed chemical kinetics modelling that PAHs can, under certain conditions, form in carbon-star molecular envelopes within a 900–1,100-K temperature regime. Although homogeneous gas-phase condensation of PAHs might lead to the formation of grains, we suggest instead that SiC nucleates at higher temperatures and provides a surface for subsequent carbon condensation. Here we present experimental evidence in support of this idea.

The present work was motivated largely by recent experimental findings that silicon carbide particles nucleate homogeneously and grow rapidly above 1,400 K from a gaseous mixture of methane and silane highly diluted in argon¹⁵. The objective of the present experiments was to determine whether SiC nucleates in an atmosphere of hydrogen. This question is not trivial, as, for example, the interstellar abundance of hydrogen suppresses entirely the formation of aromatic carbon molecules at high temperatures¹⁴.

The experiments were performed¹⁵ in a 5.71-cm inner-diameter, 3.7-m-long stainless steel shock tube. A gaseous reaction mixture was prepared from high-purity ($\geq 99.995\%$) gases. Before each experimental run, the driven section was evacuated to 10^{-3} torr and was filled with 15–27 torr of the reaction mixture. Powders formed under reflected-shock conditions were collected on amorphous carbon films at the end wall of the tube, and were characterized by transmission electron microscopy (TEM) and selected-area electron diffraction.

In the previous study¹⁵, a 3% SiH₄/3% CH₄/94% Ar mixture was pyrolysed at temperatures from 800 to 3,650 K and pressures from 0.46 to 4.16 atm. Silicon carbide particles did not form below about 1,400 K; the powder that formed consisted of silicon only. Above, 1,400 K, the powders were a mixture of β -SiC and silicon particles 20–50 nm in diameter. The SiC/Si ratio was found to increase with temperature. In the present study, pyrolysis of a hydrogen-added mixture, 3% SiH₄/3% CH₄/60% H₂/34% Ar, was carried out over temperature and pressure ranges of 1,300–2,000 K and 1.07–1.59 atm, respectively. These ranges of experimental conditions, as well as the hydrogen concentration, represent the limits achievable in the present experimental apparatus. The powders formed above 1,400 K were again a mixture of β -SiC and silicon particles 20–50 nm in diameter, and similar temperature-dependence upon the SiC/Si ratio was observed. A TEM micrograph of a typical powder and the corresponding diffraction pattern are shown in Fig. 1. From these results we conclude that the addition of H₂ to a gas mixture of silane, methane and noble gas has little effect on high-temperature formation of SiC particles.

Combining these results with those of our previous study on the growth of aromatics in H₂ atmospheres¹⁴, we are led to propose that SiC particles are the first form of carbonaceous material to condense out of carbon-star envelopes, thereby creating nucleation sites for further grain growth. This proposal is based on three specific observations: (1) in H₂ atmospheres at temperatures above $\sim 1,100$ K, the production of aromatic hydrocarbons is entirely suppressed^{14,16,17} whereas the production of SiC particles is unaffected; (2) SiC formation is extremely fast, with a rate that increases with temperature (for example, rate $\sim 10^6 \mu\text{m h}^{-1}$ at 2,800 K)¹⁵; and (3) silicon is an abundant element in stellar atmospheres.

Silicon carbide has long been proposed as an important component of interstellar grains. Molecular-equilibrium calculations^{18–20} suggested that SiC will condense out of carbon-star circumstellar envelopes at temperatures of around 1,500–1,800 K. Gilra²¹ constructed a multi-component grain model in which SiC particles 60–90 nm in size are responsible for the infrared and visual extinction of interstellar dust. This model has been criticized because such small grains may not be readily ejected by stellar radiation pressure¹⁹ and because it may require more silicon than is available. In the mid-1970s, direct observational evidence for SiC in carbon-star atmospheres emerged as the favoured interpretation of the emission feature peaking at 11.2 μm that is seen in carbon stars^{10,22–24}. McCabe²⁰ has constructed an elegant model of carbon-star envelopes in which SiC grains condense at 1,500 K, various other molecular species (such as SiO and SiS) freeze out onto the SiC grains at 1,250 K, and graphite condenses onto the grains between 1,000 and 600 K.

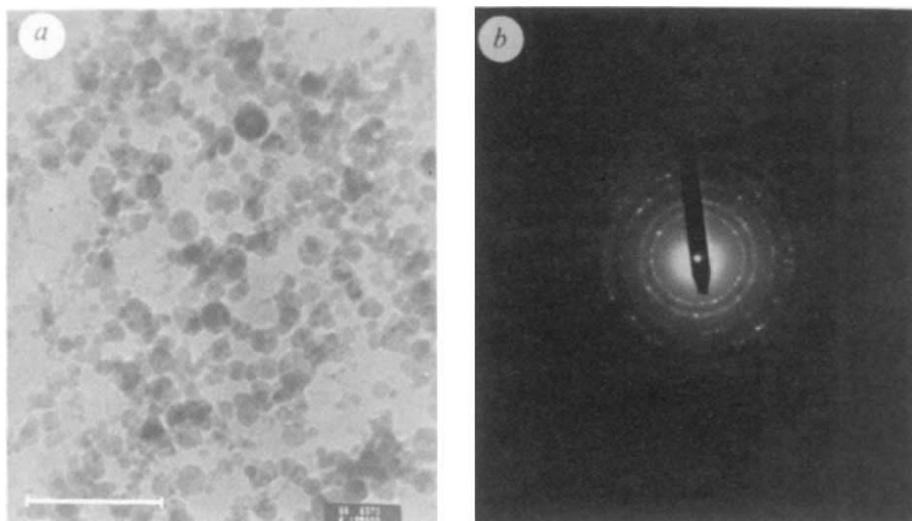


FIG. 1 *a*, TEM micrograph of powder obtained at a temperature of 1,702 K and pressure of 1.37 atm (scale bar represents 100 nm). *b*, Selected-area electron diffraction pattern of the powder in *a*. The first (innermost) ring corresponds to the Si (111) reflection, and the second corresponds to the β -SiC (111) reflection.

McCabe proposed that graphite condensation is inhibited and SiC condensation is enhanced close to the star because of an 'inverse greenhouse effect'.

We point out that the previous models for SiC condensation in stellar atmospheres may be unrealistic. First, solid SiC particles may not form directly from gas-phase SiC molecules, as assumed in equilibrium models; various intermediate species may be involved. Second, the classical nucleation theory usually applied may be extremely inaccurate. For example, recent measurements of condensation rates^{25,26} differ from theoretical predictions by as much as a factor of 10^6 . The experimental result presented here clearly demonstrates, without relying on questionable theoretical grounds, that SiC does in fact condense rapidly. We further find that the condensation rates are greatest at temperatures above 2,800 K, far hotter than theoretical predictions¹⁸⁻²⁰. Therefore we conclude that SiC should be an early and abundant condensate in cooling carbon-rich stellar envelopes, perhaps even appearing at the photosphere.

In Fig. 2 we illustrate our proposed model of carbon-grain formation. Once formed at temperatures above ~2,000 K, SiC particulates provide a surface for the heterogeneous nucleation of carbonaceous species. Evidence from laboratory studies on growth of various forms of carbon suggests that the carbon should be mostly graphitic at these high temperatures, because the sp^2 lattice of graphite is energetically favourable^{27,28}. However, in addition to energetics, structural and kinetic factors should also affect the deposition process. Given the steric sp^3 configuration of SiC, we expect a transition layer of mixed sp^3/sp^2 (that is, amorphous) carbon to appear before the formation of pure graphite. Because the energetic advantage of sp^2 over sp^3 structures is not very large²⁸, and given that chemical reaction rates should be fast at high temperatures, the growth may be dominated by kinetic rather than thermodynamic factors. Hence, the carbonaceous materials deposited on SiC nuclei at intermediate temperatures should be primarily amorphous. As the temperature falls to about 1,100 K, aromatic molecules begin to form in the gas phase¹⁴ and then condense onto the growing particles. These aromatic species are held to the grain by van der Waals forces and are likely to form 'graphitic' microstructures of turbostratic layers of PAHs similar to soot crystallites²⁹. Finally, the resulting grains may aggregate into larger structures such as are seen in soot formation. The stages of grain growth may not be as distinct as depicted in Fig. 2. If carbon stars have turbulent quasi-static inner envelopes^{14,30}, a grain may rise and fall through the regions of amorphous- and aromatic-carbon deposition a number of times.

Silicon carbide is not the only material that can nucleate in the high-temperature gas-phase environment. Silicon also exhibits fast nucleation rates, but the fractional yield of Si particles is small³¹; and those which are formed will be converted to SiC (refs 15, 32). Pure carbon clusters probably form, as indicated by the results of shock-tube pyrolysis studies^{33,34}. We do expect some grain formation to be initiated by these carbon clusters, as opposed to SiC, and in some stars this may be the dominant mechanism. In general, however, the nucleation of carbon is unlikely to be faster than that of SiC in high-temperature hydrogen environments. The carbon clusters can take the form of amorphous carbon, diamond or carbyne; these materials, for instance, nucleate homogeneously in acetylene pyrolysis³⁵ and hydrocarbon plasma³⁶. Fullerenes have been proposed⁷, but their formation is not favourable. The arguments against fullerene formation in flames³⁷ also apply to stellar conditions, where abundant hydrogen entirely suppresses the acetylene-addition steps of molecular growth above 1,100 K (ref. 14). At lower temperatures (below 1,300 K) diamond may compete with amorphous-carbon deposition on the grain surface. At even lower temperatures (below ~1,000 K), when PAHs are present in high concentrations, their homogeneous gas-phase condensation (that is, PAH molecules coalescing into a 'sandwich' cluster) may lead to the additional production of

nucleation sites. But this process should be slower than the heterogeneous condensation of aromatics on already existing SiC-based particles because of the difference in energies between aromatic-aromatic and aromatic-particle bondings, although the balance might be changed by a particularly high PAH concentration or by the presence of ions.

Many features of our model draw support from the results on diamond and diamond-like film growth in chemical-vapour-deposition processes at low pressures (see recent reviews^{28,38,39}, experimental reports^{40,41} and refs therein). It has been established that all forms of carbon—amorphous carbon, graphite and diamond—are deposited at similar experimental conditions with a close kinetic competition between them. High-quality diamond films are produced at temperatures from ~1,100 to ~1,300 K. Silicon carbide and amorphous carbon have been observed to form interlayers in deposition of diamond on silicon substrates, and diamond was shown to deposit on a SiC substrate. The nature of amorphous carbon, the subject of particular interest to the present discussion, is usually reported to have a composite character: a mixture of crystalline and amorphous sp^2 and sp^3 phases. Unfortunately, the work in this field is only now beginning to produce quantitative relationships between experimental conditions and film properties, so that the parallel between these processes and interstellar dust formation can be no more than qualitative at present.

On the basis of our model of carbon-grain formation in stellar winds, two immediate predictions can be made. First, many grains will have small cores of silicon carbide. Our experiments indicate that the initial gas-phase nucleation of SiC is fast, resulting in a large number density of nuclei and rapid depletion of one of the reactants. The size distribution of these cores,

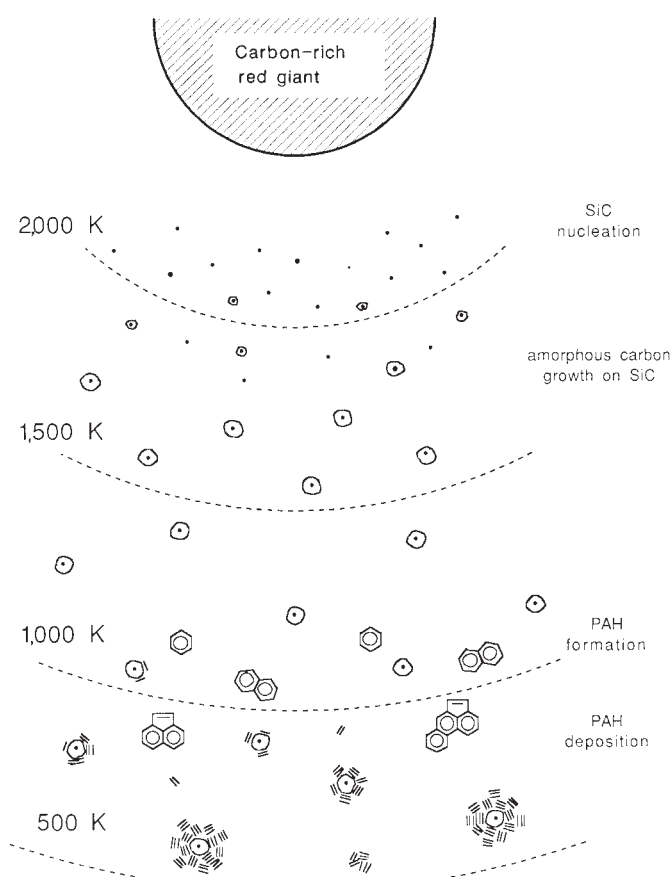


FIG. 2 Schematic illustration of the proposed model of carbon-grain formation.

however, cannot be predicted reliably from our laboratory experiments. Second, these cores will be surrounded by differing amounts and types of carbonaceous materials, depending on the characteristics of the star. In 'transition' stars, where the photospheric carbon abundance exceeds only slightly the oxygen abundance, all of the carbon will be locked in the SiC cores and no further carbon deposition will occur. In stars with higher concentrations of carbon but with conditions that do not promote the formation of PAH molecules (for example, rapid wind acceleration close to the star¹⁴), the SiC cores will be surrounded by amorphous carbon. In stars with PAH-producing envelopes¹⁴, the SiC/amorphous-carbon grains may grow substantially by deposition of soot-like structures.

The model provides explanations and motivations for certain astronomical observations. First, spatial variations in interstellar extinction features² may be due to different types of carbonaceous grains produced by carbon stars with different chemical, temperature and dynamic conditions in their molecular envelopes. Given the extreme sensitivity of PAH production to these conditions¹⁴, a single star may produce different types of grain at different times. Second, the observation that infrared-emitting 'dust' appears only beyond several stellar radii⁴² is explained by our prediction that the main carbon deposition occurs around 1,000 K, where the PAH¹⁴ and solid-carbon formation rates are maximal. Third, studies comparing the spatial distribution of the 11.2- μm SiC feature, or its spectral shape, with thermal dust emission might elucidate the relationships described here.

Our model is also consistent with recent laboratory studies^{43–46}, which identified grains of silicon carbide as a constituent of primitive meteorites. Isotopic analysis of these grains point to their formation in the atmospheres of carbon-rich red-giant stars. Specifically, the C β fraction of the Murray and Murchison C2 chondrites has been identified as 'type 1' SiC, with noble-gas isotopic ratios indicating an origin in stars undergoing slow-neutron-capture (s-process) nucleosynthesis. The bulk sample from which these SiC grains were derived also contain aromatic, amorphous and diamond forms of carbon. However, the laboratory procedures involved in isolating and analysing these grains do not permit the detailed characterization of the relationship between SiC and these forms of carbon that is required for confirmation of our predictions regarding the layering of SiC, amorphous and graphitic carbon in grains produced by carbon stars. $\square$

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Concentration of sunlight to solar-surface levels using non-imaging optics

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THE flux at the surface of the Sun, $\sim 6.3 \text{ kW cm}^{-2}$, falls off with the square of distance to a value of $\sim 137 \text{ mW cm}^{-2}$ above the Earth's atmosphere, or typically $80\text{--}100 \text{ mW cm}^{-2}$ at the ground. In principle, the second law of thermodynamics permits an optical device to concentrate the solar flux to obtain temperatures at the Earth's surface not exceeding the Sun's surface temperature. In practice, conventional means for flux concentration fall short of this maximum because imaging optical designs are inefficient at delivering maximum concentration. Non-imaging light-gathering devices can improve on focusing designs by a factor of four or more, and approach the thermodynamic limit. We have used a non-imaging design to concentrate terrestrial sunlight by a factor of 56,000, producing an irradiance that could exceed that of the solar surface. This opens up a variety of new applications for making use of solar energy.

Thermodynamics places an upper limit on the solar flux density achievable on Earth and, correspondingly, on the concentration ratio of any optical device. This limiting concentration ratio, C_{max} , is related to the Sun's angular size (2θ) by

$$C_{\text{max}} = 1/\sin^2 \theta \approx 1/\theta^2 \quad (1)$$

As $\theta = 0.27^\circ$ (4.67 mrad), $C_{\text{max}} \approx 46,000$. When the target is immersed in a medium of refractive index n , this limit is increased by a factor of n^2 :

$$C_{\text{max}} = n^2/\sin^2 \theta \quad (2)$$

This means that a concentration of about 100,000 will be the upper limit for ordinary refractive materials ($n \approx 1.5$).

However, conventional means for concentrating sunlight will fall substantially short of this limit, because imaging optical design is quite inefficient at delivering maximum concentration. For example, consider a paraboloidal mirror used to concentrate sunlight at its focus. We can relate the concentration ratio to

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the angle 2ϕ subtended by the paraboloid at its focus and the Sun's angular size:

$$C_{\text{para}} = (\sin \phi \cos \phi / \theta)^2 = \frac{1}{4} \sin^2 2\phi / \theta^2 \quad (3)$$

where we have used the small-angle approximation for θ . In equation (3) C_{para} is maximized at $\phi = \pi/4$, or

$$C_{\text{para, max}} = 1/(4\theta^2) = \frac{1}{4} C_{\text{max}} \quad (4)$$

In fact, this result does not depend on the detailed shape of the paraboloid and would hold for any focusing mirror. One fares no better (and probably worse) with a lens, because the optimum paraboloid in the above example is equivalent in concentrating performance to a lens with focal ratio $f=1$ which has been corrected for spherical aberration and coma. Such high-aperture lenses are typically complex structures with many components. The limit given by equation (1) would require a lens with focal ratio $f=0.5$, which is unattainable.

The reason for this underachievement is clear. The paraboloid causes imaging perfectly on-axis, but has severe off-axis aberration (coma) which produces substantial image blurring and broadening. The essential point is that imaging is unnecessarily restrictive when only concentration is required. Non-imaging optics¹ departs from the methods of traditional optical design to develop instead techniques for maximizing the collecting power of concentrating elements and systems. Non-imaging designs exceed the concentration attainable with focusing techniques by factors of four or more, and approach the theoretical limit (corresponding to ideal concentrators). In the 'edge ray' method¹, maximum concentration is achieved by ensuring that rays collected at the extreme angle for which the concentrator is designed are re-directed, after at most one reflection, to form a caustic on the absorber. In the 'flow-line' method², on the other hand, reflective surfaces that follow the lines of net flux, combined with refractive surfaces, can lead to 'ideal' three-dimensional concentrating elements³.

Here we describe the design and operation of a concentrator based on these principles, which can produce a solar flux equal to 56,000 'suns' (56,000 times the ambient level of sunlight) and is expected to exceed 70,000 suns in future tests.

In principle, a single non-imaging optical element could be designed that would attain the theoretical limit of concentration

C_{max} given by equations (1) and (2). However, when C_{max} is very large, such an element also becomes large and unwieldy. A more practical design is to employ a non-imaging concentrator at the focal plane of an imaging element such as a paraboloid. This non-imaging 'secondary concentrator' can provide an additional concentration factor approaching the 'ideal' limit when the acceptance angle θ is set equal to ϕ in equations (1) or (2). Combining the concentration by the paraboloid with that from the secondary concentrator ($n^2/\sin^2 \phi$), the overall concentration is

$$C = n^2 \cos^2 \phi / \theta^2 \quad (5)$$

In the limit of small ϕ , which corresponds to a large focal ratio, such two-stage concentrators can achieve concentrations quite close to the theoretical limit. We chose $\phi \approx 11^\circ$, corresponding to a parabolic mirror with focal ratio $f=2.5$. We filled the secondary with a transparent oil of refractive index $n=1.53$. From equation (5), the geometrical concentration, before taking into account absorption and blocking, is therefore

$$C = [1.53 \cos(11^\circ) / (4.67 \times 10^{-3} \text{ rad})]^2 \approx 104,000 \quad (6)$$

The approach is summarized schematically in Fig. 1a. The secondary element was designed according to the edge-ray method. Rays incident on the secondary from the rim of the primary are re-directed to the edge of the exit aperture after refraction by the curved entrance aperture and reflection by the side wall, as shown in Fig. 1b. The experimental arrangement is shown in Fig. 2a; a detailed description of the experiment is published elsewhere^{4,5}. The principal sources of loss were absorption in the oil and blocking by the calorimeter used to measure the flux (Fig. 2b). Measuring the flux reliably proved to be the most difficult aspect, because irradiance levels were beyond the range of conventional radiometry. In a series of experiments we achieved an irradiance of 4.4 kW cm^{-2} at an insolation (direct solar component) of 80 mW cm^{-2} , corresponding to a concentration of 56,000 suns.

If deployed above the Earth's atmosphere, this two-stage concentrator would give an irradiance ($\sim 7.7 \text{ kW cm}^{-2}$) in excess of that on the Sun's surface ($\sim 6.3 \text{ kW cm}^{-2}$). Simple improvements can increase the flux still further. For example, replacing the metallic oil-filled secondary with an all-dielectric secondary

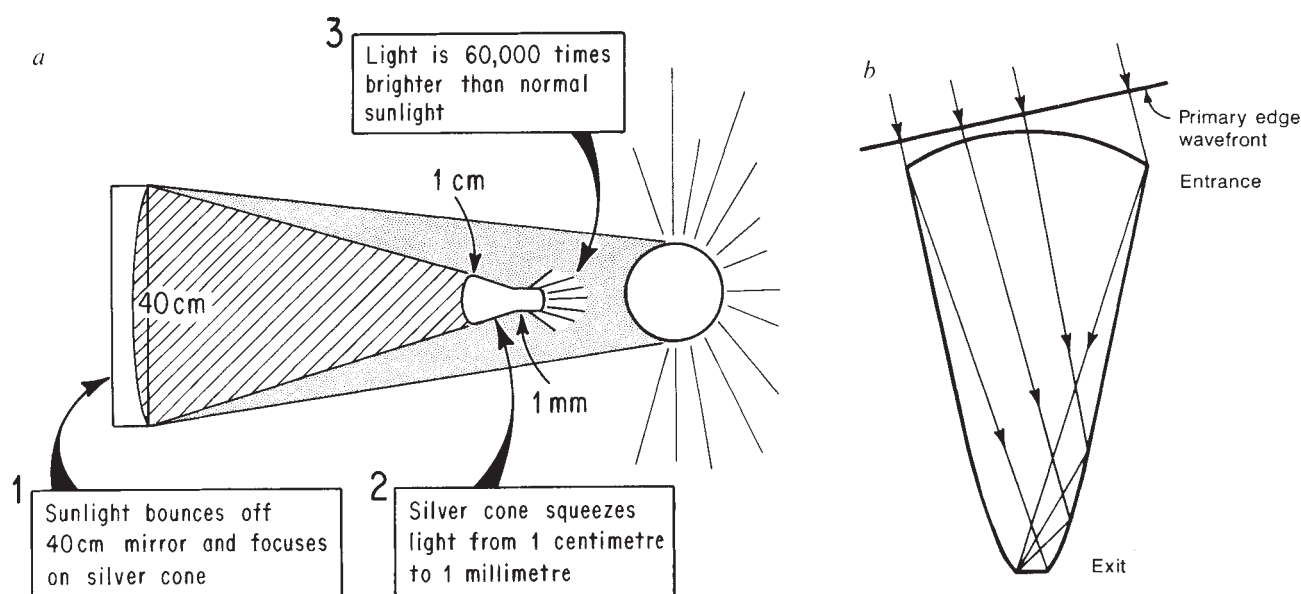


FIG. 1 a, Schematic diagram of the high-flux solar concentrator. b, The edge-ray principle. The secondary wall profile is designed in the meridional plane to image the rays from the primary edge to the secondary exit rim.

Thus the secondary concentrates all rays from the primary interior to the secondary exit aperture in the meridional plane.

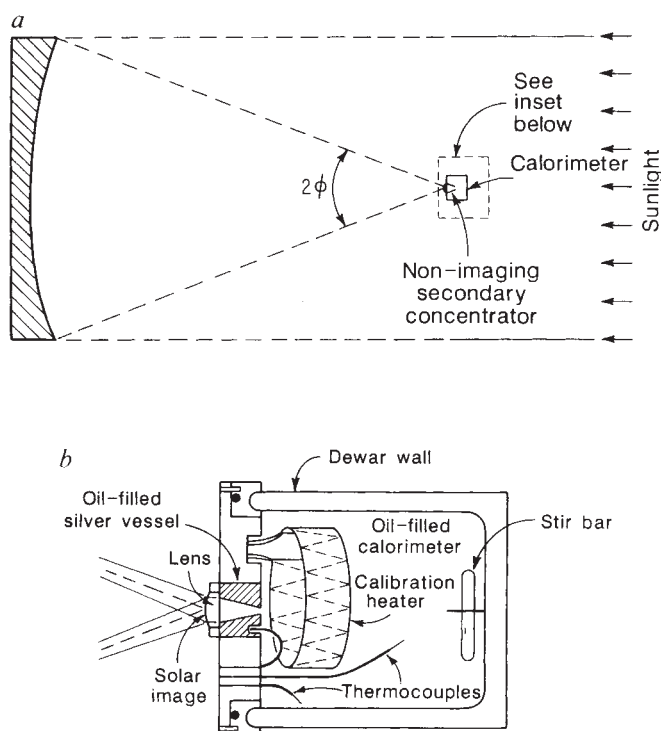


FIG. 2 *a*, Two-stage concentrator with refracting non-imaging secondary. The angle 2ϕ is subtended by the parabolic primary reflector at its focus. *b*, Oil-filled Dewar calorimeter, which measures the flux by comparison with a calibrated electrical heater.

(which operates by total internal reflection⁶) is very effective, because (1) absorption is substantially reduced by proper choice of material (sapphire, for example), (2) imperfect specular reflection is replaced by total internal reflection, and (3) concentration increases as n^2 (for sapphire, $n \approx 1.8$). Thus, one might hope to achieve concentrations of at least 80,000 suns. Even on the Earth's surface this would correspond to an irradiance substantially in excess of that on the surface of the Sun, and would be far in excess of the most intense artificial broadband continuous light sources.

Applications include the conversion of sunlight to laser light (ref. 7 and references therein). Conversion efficiencies in the past have been limited by the solar flux achievable and laser materials available^{8,9}. Materials such as alexandrite, dyes and glasses require a high solar flux to lase. Other applications of high radiant flux include the destruction of hazardous waste¹⁰ and the processing of specialized materials (for example, the production of certain fibres of high tensile strength requires very high temperatures ($\sim 3,000$ K) (R. C. Krutenat, personal communication)). Other applications should be found once the availability of this ultra-high flux becomes generally recognized.

Direct observation of shape selectivity in zeolite ZSM-5 by magic-angle-spinning NMR

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THE catalytic conversion of methanol to hydrocarbons in the gasoline boiling range¹ (30–200 °C) using zeolite ZSM-5 at ~ 370 °C has attracted much attention and is now used industrially. Methanol (CH_3OH) is dehydrated to dimethyl ether (DME) and the equilibrium mixture of CH_3OH and DME is then converted to olefins, aliphatics and aromatics up to C_{10} . However, the mechanism of the reactions involved, particularly in regard to the formation of the first carbon-carbon bond and the nature of the intermediates, remains a matter for speculation. Here we report the use of ^{13}C magic-angle-spinning NMR to identify 29 different organic species in the adsorbed phase, and to monitor their fate during the course of the reaction. This technique allows us to observe different kinds of shape selectivity in the zeolite host, to identify CO as an intermediate in the reaction, and to distinguish unequivocally between mobile and attached species. The results will assist the design of shape-selective solids and provide a better understanding of catalytic processes in the intracrystalline space.

Shape selectivity of zeolites^{2–6} arises from the fact that the probabilities of forming various products in the narrow intracrystalline cavities and channels are largely determined by molecular dimension and configuration. Three kinds of shape selectivity have been envisaged in the classic paper reporting the discovery of catalytic activity of zeolites². Reactant selectivity occurs when only certain molecules can access the intracrystalline space and react there, others being too large to enter the pores. In product selectivity only some of the various species formed within the channels and cavities can diffuse out of the crystallite to appear as reaction products. Restricted-transition-state selectivity takes place when certain reactions cannot proceed at all because they would involve transition states requiring more space than is available in the intracrystalline space. The evidence for the existence of the product and transition-state selectivities so far available is indirect, as it relies on the absence of certain species in the products rather than on the presence of others in the intracrystalline space, something which has not, until now, been directly monitored.

Zeolite H-ZSM-5 ($\text{Si}/\text{Al} = 30$) was prepared by ammonium-exchanging Na-ZSM-5 and calcination at 550 °C in air. Samples were dehydrated by heating at 400 °C for 6 h under vacuum and then 50 torr of 30 wt% ^{13}C -enriched CH_3OH was adsorbed at 20 °C. Sealed sample capsules, designed⁷ to be spun inside the magic-angle-spinning (MAS) rotor up to 3 kHz, were treated at precisely controlled temperatures in the range 20–370 °C for various lengths of time, and then quenched in liquid nitrogen. All results have been obtained using high-power decoupling only, unless otherwise indicated. MAS NMR of sealed samples gives a vast gain in resolution in comparison with earlier work^{8–14}.

The NMR spectrum of zeolite H-ZSM-5, with CH_3OH and maintained at 20 °C (Fig. 1*a*), contains a single resonance at 50.8 p.p.m. due to adsorbed CH_3OH . After heating the sample to 150 °C for 20 min, the spectrum (Fig. 1*b*) is composed of two signals, at 50.5 and 60.5 p.p.m., corresponding to CH_3OH and DME respectively. Figure 1*c* shows the spectrum of the sample treated at 250 °C. After treatment for longer than ~ 15 min a new signal appears at 184 p.p.m., due to carbon monoxide. We have confirmed this assignment, first by noting that the species responsible is stable at ambient temperature and gives rise to a single resonance; second, by acquiring the spectrum without

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proton decoupling to find that the signal is unsplit, that is, no J -coupling to protons is present); third, by noting that the signal disappears when the spectrum is measured with cross-polarization, indicating that it corresponds to a mobile rather than attached species and/or an unprotonated carbon; and fourth, by reference to the literature¹⁵.

After heating at 250 °C for 54 min, the sample was heated to 300 °C. After 10 min at 300 °C the spectrum (Fig. 1d) contains signals from various aliphatic carbons as well as CH₃OH, DME and CO. Figure 2 shows the spectrum of a sample treated at 300 °C for 35 min. CH₃OH and DME have been completely converted to a mixture of aliphatics and aromatics. Although some signals overlap, particularly those from methyl groups attached to aromatic rings, all could be reliably assigned because most compounds give rise to several NMR peaks. The two peaks with negative chemical shifts are due to cyclopropane and methane¹⁶. Because of the mobility of the two species, these signals are not observed when cross-polarization is used. When the spectrum is acquired without decoupling (see inset) the signal at -10.7 p.p.m. is split into a quintet with the individual components in the intensity ratio close to 1:4:6:4:1 with $J_{CH} = 124$ Hz, characteristic of methane; the signal at -6.1 p.p.m. is split into a triplet, with $J_{CH} \approx 100$ Hz. The fact that the spectrum obtained without decoupling is only slightly broadened indicates the relatively fast motion of all species present. A careful choice of NMR experiment thus allows us to distinguish between adsorbed and mobile species.

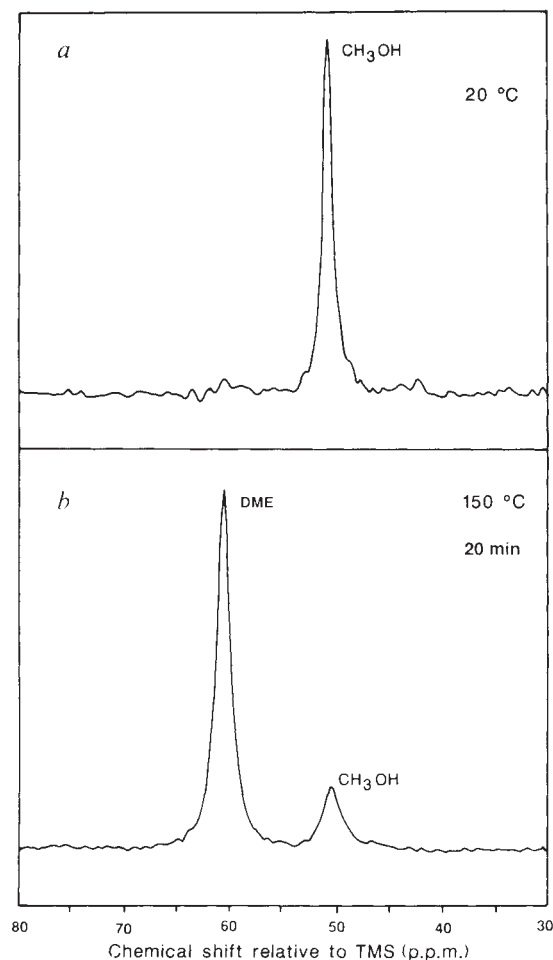
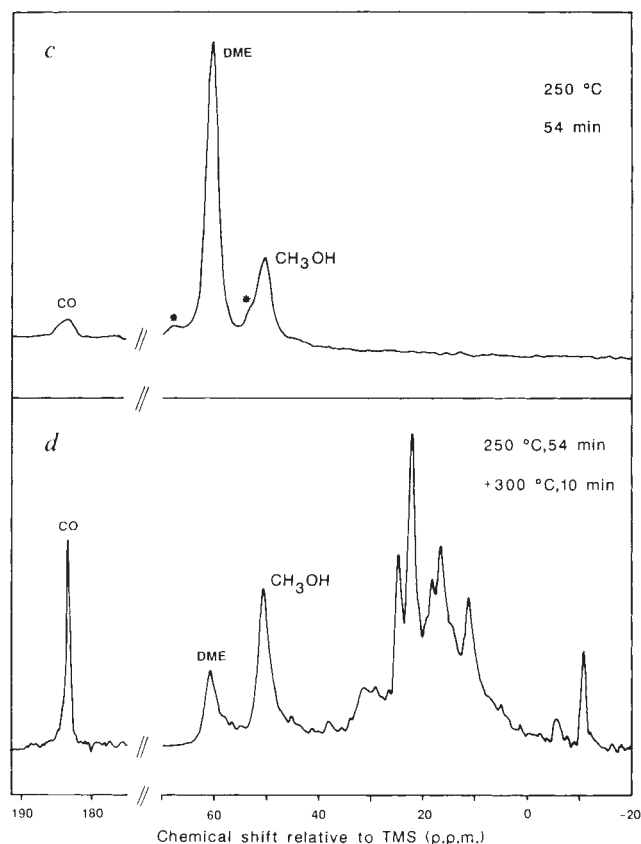


FIG. 1 ^{13}C MAS NMR spectra (proton decoupling only) of H-ZSM-5 with 50 torr of adsorbed CH₃OH and recorded at room temperature. a, No heating; b, 150 °C for 20 min; c, 250 °C for 54 min; d, 250 °C for 54 min, followed by 300 °C for 10 min. Experiments were done at room temperature on a Bruker MSL-400 spectrometer. High-power decoupling experiments were done with

The distribution of adsorbed species in the sample treated at 300 °C is very different from that observed in the reaction products (Table 1). The principal aromatics expected to be present⁸ are *m*- and *p*-xylene, 1,2,4-trimethylbenzene and toluene. However, the main species actually found in the adsorbed phase are *o*- and *p*-xylene and 1,2,4,5-tetramethylbenzene, with smaller amounts of 1,2,4-trimethylbenzene and 1,2,3,5-tetramethylbenzene. *m*-xylene and tri- and tetramethylbenzenes are also found but in smaller amounts. The distribution of the three trimethylbenzenes in the adsorbed phases reflects the thermodynamic equilibrium distribution (Table 1). The fact that 1,2,3- or 1,3,5-trimethylbenzenes (with kinetic diameters of 6.4 and 6.7 Å, respectively) are not found among the products, but are present in the adsorbed phase, whereas the smaller 1,2,4-trimethylbenzene (6.1 Å) is found in both, clearly demonstrates the reality of the concept of product selectivity. The channel dimensions of ZSM-5 are¹⁷ 5.6 Å × 5.3 Å, but more space is available at the intersection of the straight and zig-zag channels. The greater amplitude of thermal vibrations of the framework, which increases the maximum effective size of the channels, allows the smaller isomer to diffuse out of the crystal. The two larger isomers, however, although formed, are unable to diffuse out at 300 °C and must isomerize to 1,2,4-trimethylbenzene.

The distribution of the tetramethylbenzenes in the intracrystalline space is unexpected. None have ever been reported in the products of the reaction at 300 °C and yet all three are clearly present in the adsorbed phase. Because of the restricted



40° ^{13}C pulses and a 10-s repetition time. Experiments without higher-power decoupling used the same acquisition parameters. Cross-polarization²² (CP) spectra were acquired at 100.613 MHz with a 4.5-μs proton 90° pulse, a 2-ms contact time and a 5-s repetition time. Chemical shifts are referred to external tetramethylsilane (TMS). Asterisks (*) denote spinning sidebands.

TABLE 1 Distribution of aromatics in the reaction products (from gas chromatography) and in the adsorbed phase (from ^{13}C NMR).

Compound		Distribution		Reaction products by gas chromatography†		Adsorbed phase by ^{13}C NMR‡	
		Statistical*	Thermodynamic ²¹	300 °C ^b	370 °C ²⁰	300 °C	370 °C
toluene		—	—	m	s	w	m
xylene	<i>o</i> -	2	2	2.0 (w)	2.0 (m)	s	s
	<i>m</i> -	2	4.9	(m) } 7.5	5.1 (s)	w	s
	<i>p</i> -	1	2.2	(m) }	2.2 (m)	s	m
trimethylbenzene	1,2,4-	12	12	12.0 (m)	12.0 (m)	m	m
	1,2,3-	5	1.3	—	1.0 (w)	w	m
	1,3,5-	2	4.3	—	2.3 (w)	w	w
tetramethylbenzene	1,2,4,5-	1	1	—	1.0 (w)	s	w
	1,2,3,4-	2	0.44	—	0.5 (w)	w	vw
	1,2,3,5-	2	1.5	—	0.9 (w)	m	vw
pentamethylbenzene		—	—	—	vw	—	w
hexamethylbenzene		—	—	—	—	—	w

* Expected for random methyl substitution on the benzene ring.

† Normalized to the thermodynamic equilibrium distribution for *o*-xylene, 1,2,4-trimethylbenzene and 1,2,4,5-tetramethylbenzene. s=strong, m=medium, w=weak, vw=very weak.

‡ This work.

intracrystalline space they can only form at channel intersections, but (unlike the trimethylbenzenes) they are not generated in the thermodynamic equilibrium distribution. On thermodynamic grounds, 1,2,3,5-tetramethylbenzene (6.7 Å)^{18,19} should be the dominant species (Table 1); in fact it is 1,2,4,5-tetramethylbenzene (6.1 Å) which dominates. The thermodynamically least-favoured isomer, 1,2,3,4-tetramethylbenzene (6.4 Å), is found in small quantities. The fact that tetramethylbenzenes are not found in the products again demonstrates product shape selectivity. Their relative abundance in the adsorbed phase, on the other hand, shows that an additional type of shape selectivity occurs within the intracrystalline space. It does

not rely on the ability of species to enter or leave the crystal, nor on the size of the transition state; isomerization is sterically restricted within the crystallite at the active site itself. Selectivity of this kind is similar to the action of enzymes.

Low-molecular-weight compounds dominate in a sample treated at 370 °C in both the adsorbed and gas phases (Table 1). There is more *m*-xylene and toluene and much less 1,2,4,5-tetramethylbenzene than in the 300 °C sample. Small amounts of penta- and hexamethylbenzene are also found. At 370 °C all possible methyl-substituted benzenes up to C₁₁ have been observed²⁰ in the products. The larger species are now able to leave the crystallite because of the increased effective channel diameter. As gas chromatography shows that the distribution of trimethylbenzenes in the products is very close to that expected on thermodynamic grounds, it may appear, without the insight provided by NMR, that shape selectivity is absent. However, the spectra clearly show that a two-step shape selection is at work. First, steric effects restrict the formation of the largest 1,3,5-isomer in the intracrystalline space. Second, diffusion effects allow only the smallest 1,2,4-isomer to diffuse out of the particle to any great extent, although the 1,2,3-isomer is present in the adsorbed phase in comparable amounts.

MAS NMR can probe directly the role of the active site in shape-selective catalytic reactions on zeolites. The kind and quantity of chemical species present inside the particle can, for the first time, be directly monitored. This information, not forthcoming from other techniques, is usefully compared with the composition of the gaseous products to give new insights into

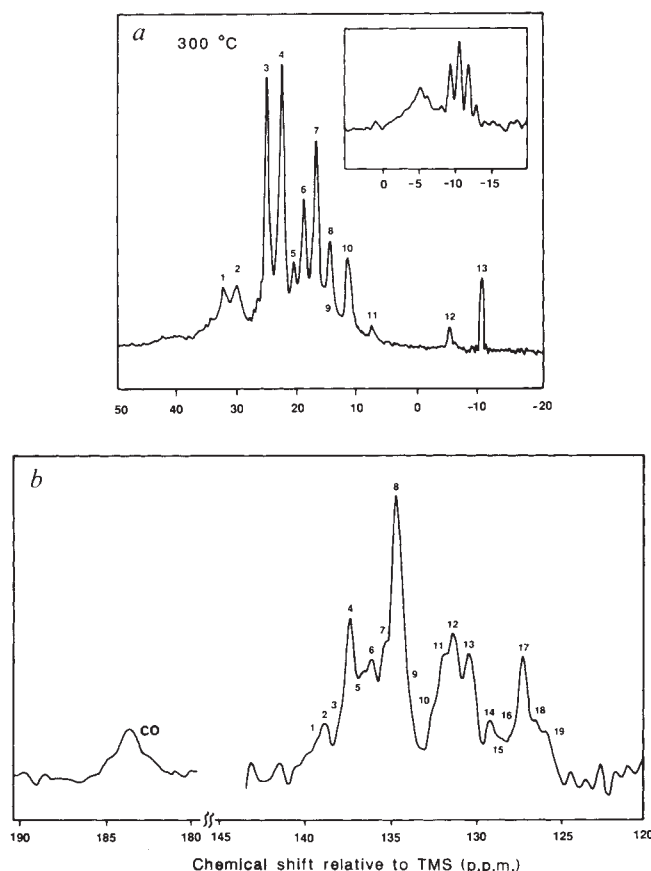


FIG. 2 ^{13}C MAS NMR spectra of a sample heated to 300 °C for 35 min and recorded with proton decoupling only. a, Aliphatic region; b, aromatic and CO region (intensities not on the same scale). The inset shows *J*-coupling of methane and cyclopropane carbons (recorded without decoupling). Spectral assignments are as follows (peak numbers and intensities (s=strong, m=medium, w=weak) are in brackets, {} for aliphatic and () for aromatic): *i*-butane {3, s}; propane {7, s}; *n*-butane {3, 9, m}; *n*-hexane {1, 4, 8, m}; *i*-pentane {1, 2, 4, 10, m}; *n*-heptane {1, 2, 4, 8, m}; methane {13, m}; ethane {11, w}; cyclopropane {12, w}; *o*-xylene (4, 12, 17, s) {5}; *p*-xylene (7, 13, s) {6}; *m*-xylene (2, 12, 15, 17, w) {5}; toluene (2, 13, 14, 18, w) {5}; 1,2,4-trimethylbenzene (4, 6, 8, 11, 13, 16, m) {5, 6}; 1,3,5-trimethylbenzene (3, 16, w) {5}; 1,2,3-trimethylbenzene (5, 7, 16, 19, w) {5, 8}; 1,2,4,5-tetramethylbenzene (8, 11, s) {6}; 1,2,3,5-tetramethylbenzene (5, 7, 10, 13, m) {5, 8}; 1,2,3,4-tetramethylbenzene (7, 9, 16, w) {5, 8}. Because of the different Overhauser enhancements of different carbons, peak intensities give only an approximate concentration of the various species. The spectrum of a sample treated at 370 °C (not shown) allows another 7 organic species to be identified.

reaction pathways on molecular sieves and to assist the design of new shape-selective catalysts. □

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Magnetofossil dissolution in a palaeomagnetically unstable deep-sea sediment

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MAGNETOFOSSILS¹, the fossil remains of bacterial magnetosomes², are found in various deep-sea sediments, and have been linked to the preservation of stable natural remanent magnetization in many of them^{1,3–5}. They have also been extracted and identified from lithified carbonates of Jurassic⁵ and Precambrian⁶ age, showing that magnetotactic bacteria have been a sedimentary source of fine-grained magnetite for much of geological time. Some clay-rich deep-sea sediments, however, do not record a stable remanent magnetization, for unknown reasons. Here we report results from a high-resolution transmission-electron-microscope study on samples of this type collected by the Deep Sea Drilling Project (DSDP); the material contains a complex mixture of single-domain magnetic minerals. Well-preserved magnetofossils show the same crystal structures as those found in magnetosomes from recent bacteria^{7–10}, whereas others in these same preparations display a wide range of dissolution, corrosion and aggregation effects. Rock magnetic measurements are consistent with the presence of these alterations in many DSDP sediments, including those that preserve reliable palaeomagnetic directions. As we were unable to find authigenic magnetic minerals in our sample, and as the magnetic fraction is dominated by well preserved magnetofossils, we suggest that the poor preservation of the magnetization is a result of diagenetic interactions between the magnetofossils and the clay minerals in the matrix.

Sediment cores collected on DSDP Leg 73 yielded some of the best magnetostratigraphic records yet obtained¹¹, and many of the sediment samples from these cores have yielded abundant magnetofossils^{1,3,5,12}. However, the Miocene interval from 25 to 50 m depth at site 522, which is clay-rich, gave magnetostrati-

graphic results that were difficult to reconcile with the geomagnetic polarity timescale¹¹. This section of core is therefore promising for identifying processes that can alter or mask the original natural remanent magnetization (NRM) of sediments. We selected samples for high-resolution transmission electron microscopy (HRTEM) from cores 522-12 and 522-15 within this interval. For comparison, we also obtained freeze-dried samples of the magnetotactic bacterium *Aquaspirillum magnetotacticum*, and synthetic magnetite formed from the inversion of green rust (K. Kuma *et al.*, manuscript in preparation), the mechanism thought to be responsible for the production of authigenic magnetite in soils^{13–15}.

We used the technique of von Döbeneck *et al.*^{3,16} to extract the sub-micrometre grain-size fraction. The magnetic extracts were examined on a Philips 430 300-keV microscope with an analytical pole piece, a 2-Å line resolution, a double-tilt side-entry specimen holder, and an energy-dispersive X-ray analysis system. Images were made using objective apertures of 100 µm, under-focused 60–240 Å into the particles. Electron microdiffraction and selected-area diffraction were used to make accurate measurements of lattice spacings and crystallographic orientation. In addition to X-ray diffraction analysis, precise determinations along the magnetite/maghemite solid-solution series were made, by reference to an internal gold diffraction pattern from a thin film deposited on the sample⁵, and confirmed that the magnetic particles consisted of the mineral maghemite (cubic; cell constant $a_0 = 8.3440 \pm 0.014$ Å), which is a common low-temperature, topotactic oxidation product of magnetite. Classification of the particles as single-domain or superparamagnetic is based on measured sizes and shapes according to the analysis of Butler and Banerjee¹⁷.

Several distinct types of single-domain particle are present, some of which are found in small chains or clumps of similar particle types (Fig. 1). A new type of single-domain particle is shown on the left-hand side of the stereo pair in Fig. 1a and b (arrow 1; enlarged in Fig. 2d), which occurs as a tangled, interlocking mesh. The overall dimensions of these particles, measured by the extent of coherent lattice fringes (Fig. 2d), place them within the single-domain stability field¹⁷. Energy-dispersive X-ray analysis reveals the presence of ~1–2% titanium. The right-hand side of Fig. 1a and c (arrow 2 in both cases) shows well preserved crystals of shape and size similar to those produced by a variety of magnetotactic bacteria^{5,6,16} (therefore termed magnetofossils). The corresponding images in Fig. 2a, b and c show that the crystals are perfect except for weak irregularities along the outlines. Several progressive stages of alteration within magnetofossil chains can be seen in Fig. 1c and the enlargement in Fig. 1d. Long chains of magnetofossils with superparamagnetic particles on their surface are present in the sample (crystals on the right in Fig. 1c and d), as well as bead-like chains of apparently superparamagnetic-sized particle aggregations. However, the image of Fig. 2e reveals that these are not aggregations of smaller particles, but are in fact chains of single crystals, each of which contain parallel sets of lattice fringes. Energy-dispersive X-ray analysis on all of these particles, as well as the magnetofossils, does not reveal titanium.

Electron-microscope examination of the magnetic fine fraction from this deep-sea sediment therefore reveals three distinctly different morphological types of single-domain particle. The first and easiest to distinguish are the magnetofossils shown in Fig. 2a–c. Our observation that these particles have crystal structures similar to those of recent magnetosomes in living bacteria^{2,7–10} suggests such an origin. The oxidation of the original magnetite to the solid-solution endmember, maghemite, does not introduce visible crystal defects. Electron microscopy, however, does reveal slight preferential alteration along the [100] directions, which is not apparent from the lower-magnification images of Fig. 1a and b.

The second particle type includes the tangled, interlocking crystals which lack regular habit and contain 1–2% Ti. These

cannot be derived from the larger fragments of titanomaghemites in this sample, which have much higher Ti concentrations³, nor from the magnetofossils, which lack Ti. Although these particles show some similarity to the bullet-shaped magnetofossils (arrow 2 on Fig. 1c), a comparison of microscope images shows that they are quite distinct. We have found occasional single-domain titanomagnetites (or titanomaghemite) embedded in small siliceous particles; they resemble other single-domain titanomagnetites in basalts¹⁸ and feldspar inclusions¹⁹, but are morphologically distinct from the tangled, interlocking particles. These particles are also distinctly different from authigenic magnetite found in soils^{13,14} or synthetic magnetites formed under controlled conditions²⁰. As trace levels of Ti are commonly found in magnetites from a variety of igneous rocks¹⁹, a lithogenic source is the most likely origin for these particles.

The third class of particles includes those which have, in low magnification, the appearance of an aggregation of smaller particles. One possible mechanism for producing these particles is abiotic authigenesis¹⁵; few studies of authigenic magnetic mineral formation have, however, been made on marine sediments. Karlin *et al.*²¹ report the apparent authigenic formation of magnetite in the upper 30 cm of suboxic marine sediments based on rock magnetic changes, but do not report TEM morphological observations. Other reports of authigenic magnetite in sediments reveal spherical aggregates of up to 20 μm in size^{22,23}, which are totally unlike the particles in our sample. Single-domain magnetite particles similar in size to magnetofossils have been found in soil environments^{13,14}, where they are presumably formed by the topotactic oxidation of the ferrous material green rust (refs 14, 18 and K. Kuma *et al.*, manuscript in preparation). For comparison, we therefore observed in the microscope magnetite formed by oxidation of synthetic green rust. We found that this material has a range of particle sizes and morphologies that is very different to that expected for magnetosomes. Crystal sizes range from 50 to 1,500 Å, with the larger crystals often showing overlap textures and irregular outlines. Many crystals are thin and plate-like, with offset fringes indicating crystal defects (Fig. 2f). We have never seen such structures in our DSDP samples.

A second possible mechanism is the extracellular precipitation of magnetite particles produced by growth of an anaerobic

bacterium such as GS-15 (ref. 24). However, GS-15 particles are generally much smaller than the magnetic particles in our samples, and have highly variable crystal morphologies. Furthermore, magnetite formed in the presence of these organisms has been observed only in laboratory cultures, and it is not known whether it appears also under natural conditions.

A third possibility is that these altered grains are corroded magnetofossils. We favour this interpretation for several reasons. First, when viewed in the microscope we found that the lattice fringe patterns usually extend across the entire particle, implying that it was originally a single crystal. The relict outlines as traced by the fringe patterns are similar to those of unaltered magnetofossils. Surface irregularities produced by alteration are probably responsible for the aggregate-like texture. Second, these particles are usually strung together in chains of up to several dozen particles, as are magnetofossils³⁻⁶. Third, we have found progressive alteration stages within a single chain which range from euhedral magnetofossils through to strongly damaged crystals that resemble the aggregate morphology; an example is shown in Fig. 1d. The presence of both pristine and altered magnetofossils in the same sample, sometimes within the same magnetofossil chain, probably arises through a differential dissolution process.

This dissolution process may have an interesting mechanism. Magnetotactic bacteria are known to produce iron-chelating compounds called siderophores²⁵ which are capable of dissolving even the most stable iron oxide minerals. We have observed partially dissolved magnetosomes in surface muds of freshwater lakes that contain dense populations of magnetotactic bacteria^{5,12}, long before they have oxidized to maghemite. Virtually all of this dissolution happens in this top zone; we do not see increasing dissolution with greater depth. Marine magnetotactic bacteria probably possess similar siderophore systems. Hence, variable alteration textures of this sort found in our DSDP samples probably arise from initial biochemical variations within the bacterial magnetosomes which protect some particles more than others from attack by siderophores. In this model, dissolution probably happens before particles are immobilized during compaction; that is, before the sediment would normally acquire a stable natural remanent magnetization.

Although extensive dissolution effects are present in only a small fraction of the magnetofossils in our DSDP samples, they

FIG. 1 *a, b*, Low-magnification stereo images of typical sub-micrometre sized magnetic particles extracted from sample 522-15, with 25° tilt difference. These show several morphological shapes characteristic of single-domain bacterial magnetite, including equant octahedra, elongate prisms and 'bullets'. Notice that the slight change in tilt of the specimen can yield a large difference in the projected shape of some particles, particularly the changes from diamond to hexagonal outlines which are produced by different stereographic projections of an octahedron. When viewed in stereo, the particles on the left form an interlocking, tangled mesh. *c*, Particles displaying a wide range of dissolution effects, ranging from clearly recognizable magnetofossils to strings of highly corroded particles. Scale bar in *c* is for *a, b* and *c*, and corresponds to 0.1 μm . Arrows indicate areas enlarged in Fig. 2. Higher magnification of a section of *c*. The four magnetofossils in the bottom right display dissolution increasing towards the centre of the figure. The two small arrows on the right-hand magnetofossil show octahedral vertices (viewed edge-on the [100] direction) along which dissolution or corrosion begins; the small arrow on the central particle shows that [100] alteration progresses into the interior of the particle, yielding textures similar to those of the apparent aggregates.

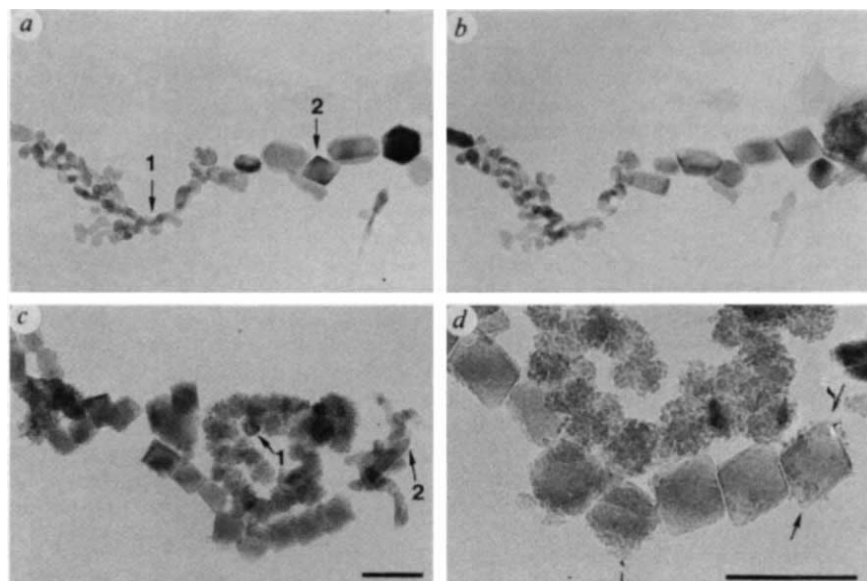
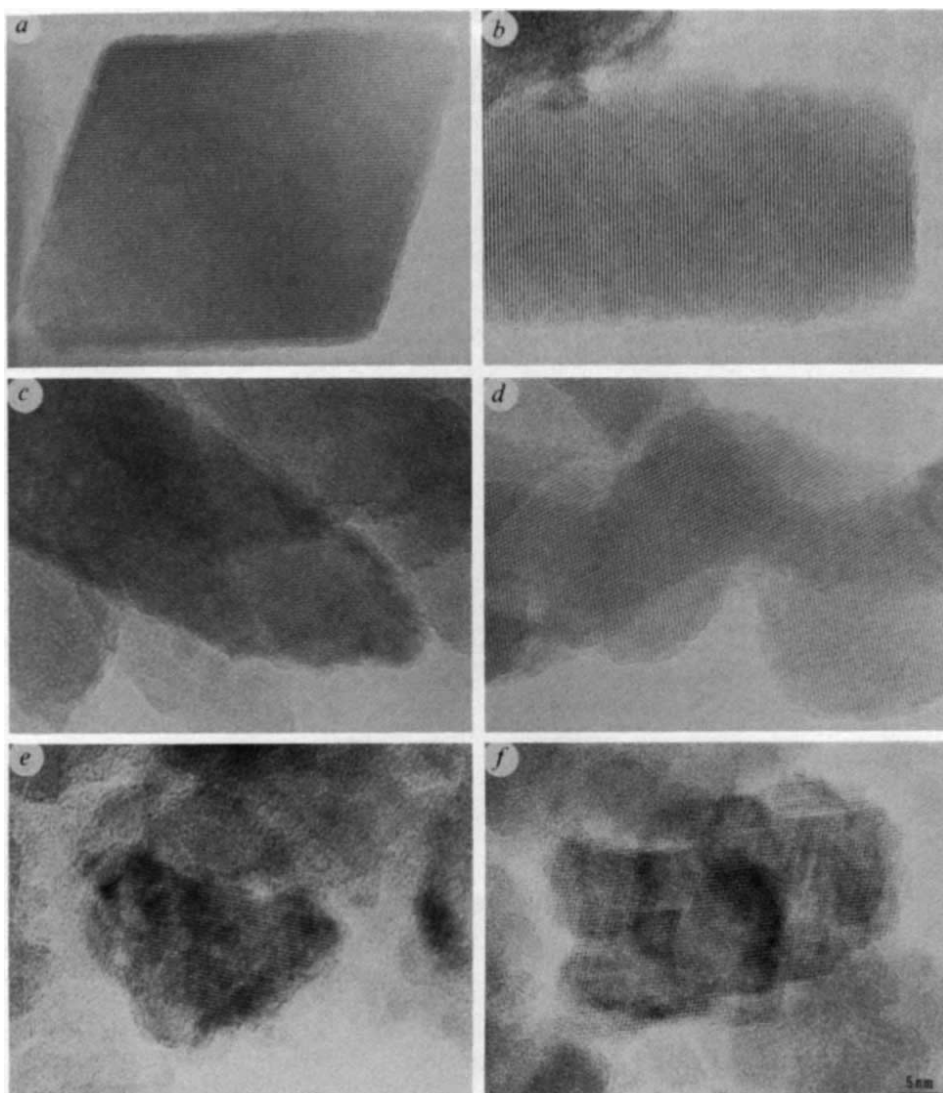
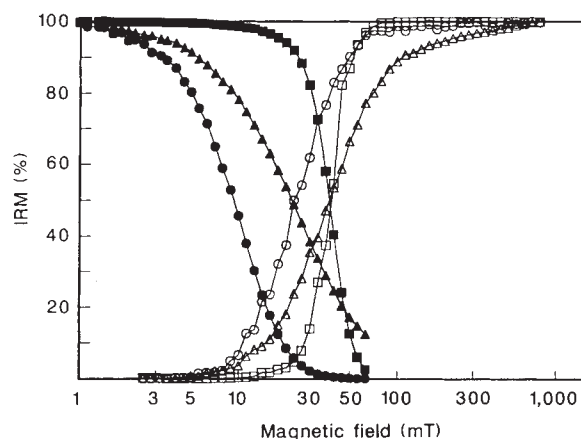


FIG. 2 Electron-microscope images of single-domain particles, locations of which (with the exception of *b*) are indicated on Fig. 1*a* and *c*. *a*, *b* and *c* are magnetofossils with weakly dissolved surfaces. The {111} lattice fringes on all three images are spaced at 4.8 Å and show no defects. *a*, Diamond-shaped particle (arrow 2 in Fig. 1*a*) viewed down the [110] axis, with two sets of intersecting {111} lattice fringes (the measured intersection angle is close to 71°). No defects in the crystal structure can be seen. Two of the octahedral vertices seen in this image (lower left and upper right) show evidence of rounding along the [100] and equivalent directions. *b*, One end of a 1,200-Å-long prismatic magnetofossil, showing that the {111} lattice fringes are perpendicular to the long axis of the crystal. Note the presence of outline irregularities perpendicular to the long axis of the particle, whereas the {111} face at the end of the particle is smooth, as were the {111} and equivalent octahedral faces of the particle in *a*. *c*, The tip of a bullet-shaped magnetofossil (arrow 2 in Fig. 1*c*), viewed in the [011] orientation and showing one set each of {111} and {200} fringes (respectively, those running from upper left to lower right with 4.8-Å spacing, and the nearly horizontal lines with 4.2-Å spacing). Note that all three of these magnetofossils show less rounding and irregularity along {111} edges than do the {100} faces. *d*, Electron-microscope image of tangled, interlocking magnetic particles (arrow 1 in Fig. 1*a*). Lattice fringes with 4.8-Å spacing are again from {111} planes without crystal defects as in the magnetofossils, but those from particles with overlapping images (judged from the stereo pair of Fig. 1*a* and *b*) intersect at the 71° angle expected for sets of {111} fringes within the same crystal. Unlike the magnetofossils of *a*–*c*, the surface outline irregularities appear to be as strong on the {111} edges as in other orientations. *e*, Electron-microscope image of a highly dissolved magnetofossil (arrow 1 in Fig. 1*c*). The nearly horizontal set of lattice fringes are {111} planes and the vertical set are {220}. The crystal outline as traced by the extent of the lattice fringe pattern is similar to



those of magnetofossils, except that the degree of crystal damage is extreme. *f*, Electron-microscope image of a thin, plate-like crystal of synthetic magnetite produced by the oxidation of green rust (sample courtesy of G. Arrhenius). Note the two sets of {111} lattice fringes and numerous defects. Scale bar in *f*, corresponding to 50 Å, applies to all images.

FIG. 3 Coercivity distribution from intact, freeze-dried cells of *A. magnetotacticum* (squares), from NaOH-extracted *A. magnetotacticum* crystals (circles), and from DSDP sample 522-15 (triangles). Open symbols show data from the isothermal remanent magnetization acquisition experiments, whereas solid points are from the corresponding one-axis alternating-field (Af) demagnetization. For the freeze-dried bacterial sample, which has non-interacting magnetosome chains, the isothermal remanent-magnetization-acquisition curve (open squares) is nearly symmetrical with respect to the paired Af demagnetization pattern (solid squares), indicating a narrow range of coercivities (25–50 mT, with a median value of 35 mT), with a nearly 'ideal' crossover point (*R* value) of 49%. In contrast, sample 522-15 (triangles) has a much broader, asymmetrical coercivity spectrum (5–100 mT) and a lower *R* value of 38%. The increase of isothermal remanent magnetization above 100 mT in this sample is probably a result of the oxidation of the magnetic minerals. Interparticle interaction effects probably influence the low *R* value, as can be seen from magnetization-Af results of purified bacterial magnetites from *A. magnetotacticum* (samples from R. P. Blakemore as used in ref. 27) (circles). These interactions raise the effective magnetization coercivities while lowering those from the Af treatment, causing a drop in the *R*-value intersection²⁸. Results from our sample lie between those from these bacterial examples, suggesting a moderate level of interparticle interaction, with perhaps some weakening of the coercivity spectra through particle corrosion.



ought to be present in other samples as well. Measurements of coercivity distributions (Fig. 3) indicate that our sample contains a coercivity range typical of other magnetofossil-rich DSDP-522 sediments that preserve stable natural remanent magnetization directions¹¹. All of these sedimentary coercivity spectra have broader distributions than those of magnetosomes in recent magnetotactic bacteria, as would be expected on the basis of corrosion and particle clumping. Furthermore, the low-temperature oxidation of magnetite to maghemite should not alter the relative direction of a crystal's magnetic moment, because these minerals are solid-solution endmembers and the particles are of single-domain size. This is consistent with the observation that maghemite magnetofossils preserve the stable natural remanent magnetization elsewhere in DSDP core 522 (refs 3, 5). We therefore conclude that the poor preservation of

the natural remanent magnetization in our DSDP samples is not a problem with the magnetic minerals.

Compared with other parts of the core, the Miocene interval from DSDP site 522 has an anomalously high clay content (over 50%). We suggest that post-depositional interactions between the sedimentary clay matrix and the single-domain magnetites may be responsible for the poor palaeomagnetic results from this portion of the core. The ultrastructure of clay-rich sediments depends strongly on the electrolyte composition in the surrounding pore waters²⁶. Structural rearrangement of the clays during diagenesis, for whatever cause, could physically disorientate adsorbed magnetic crystals of similar size. This model predicts that clay-rich sediments that possess magnetic particles much larger than magnetofossils should be better for palaeomagnetic study. □

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Tomographic image of the magma chamber at 12°50' N on the East Pacific Rise

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THE East Pacific Rise at 12°50' N (Fig. 1) is a fast-spreading ridge with intense hydrothermal activity¹, and ophiolite studies² and thermal modelling³ indicate that this is a likely setting for a magma chamber. A recent seismic-reflection experiment⁴ imaged the top of the magma chamber at this site, at a depth of 1.4 km below the sea floor, and found that it is continuous for tens of kilometres along the rise axis. Here we examine a large set of accurate P-wave travel times from a detailed seismic refraction experiment at the same site⁵. The patterns observed in the travel times demonstrate that a zone of low seismic velocities exists beneath the rise axis throughout the region studied. The best-fitting two-dimensional structure, obtained from linear inversion of the travel times, includes an axial low-velocity zone (magma chamber) only ~6 km wide, in which velocities are depressed by more than 0.5 km s⁻¹.

The MAGMA expedition⁵ collected an extensive set of seismic-refraction data at this site to determine the size and shape of the low-velocity zone associated with the magma chamber. Subsets of these data were analysed and indicated the presence of a crustal, axial low-velocity zone^{5–7}. The completion of the processing of the entire data set has yielded a large number of P-wave travel times that sample the rise-axis crustal structure in three dimensions. Explosive sources were recorded by ocean-bottom seismometers at three sites (on axis, and at 7 and 16 km east of the axis), and a total of 2,575 unique source–receiver

P-wave travel times have been measured from the seismograms. Sea Beam coverage in the area is excellent, and gridded bathymetry is now available from the University of Rhode Island, allowing complete three-dimensional bathymetric corrections to the P-wave travel times. We determined the phase velocity as a function of range for each of the three sites, and then searched the digitized sea floor beneath each shot for the point of least total travel time. This location was used as the ray starting point and the water path time was subtracted from the total travel time.

A laterally homogeneous 'average' velocity model was constructed, based on the bathymetrically corrected data from all three sites, and was used to calculate residuals (recorded travel times minus travel times calculated for a model). The velocity

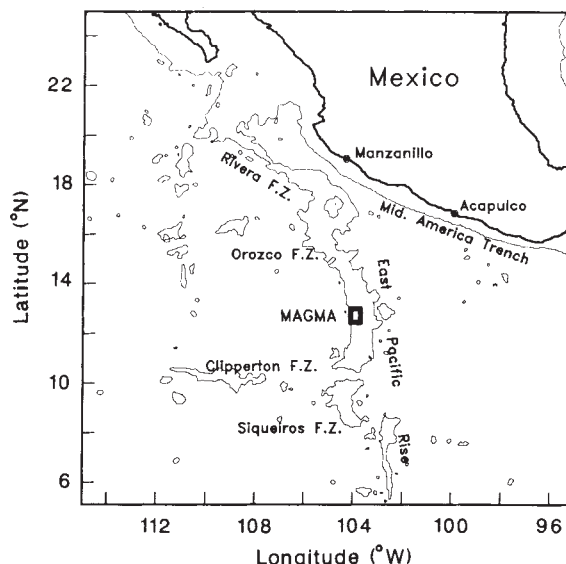
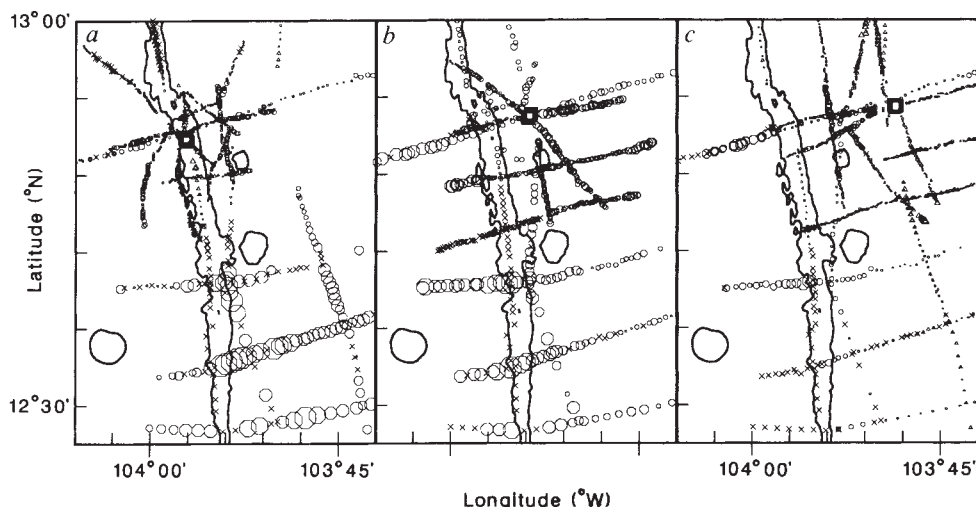


FIG. 1 The East Pacific Rise off the coast of Mexico, outlined by the 3,000-m depth contour. The box indicates the location of the MAGMA expedition.

FIG. 2 Aerial views of the travel-time residuals recorded at each of the three receiver sites; *a*, the axis site; *b*, the 7-km site; *c*, the 16-km site. The residuals are corrected for bathymetry and shown at the point where the ray enters the sea floor. The 2,675-m contour outlines the rise axis. Bold squares mark the ocean-bottom seismometer locations. Triangles represent early arrivals and circles represent late arrivals. The height of the symbol is proportional to the size of the residual; the largest circle is ~ 0.5 s. The crosses represent low-amplitude arrivals whose arrival times could not be measured.



model is smooth, contains no triplications, and is representative of normal oceanic crust, fitting the data from the 16-km site (on older crust) quite well except for rise-crossing paths. The model also fits the close-range data from the axis site. Figure 2 shows the aerial distributions of the travel-time residuals for the three sites, plotted where the ray path entered the sea floor beneath each shot; triangles indicate early arrivals, circles late ones, and the symbol height is proportional to the residual size (the largest is ~ 0.5 s). It was not possible to choose a first arrival for 443 unique source-receiver pairs, because of low signal levels rather than high noise levels. These paths are included in the diagrams as crosses, and most represent shadows of the axial low-velocity zone⁷.

All travel times recorded at the intermediate site are late with this model, indicating lower velocities immediately beneath this site. This near-axis age dependence in the shallow crustal velocities was previously observed in a subset of the data⁵, and has been observed elsewhere⁸. The behaviour is consistent with the hypothesis⁵ that the upper crust fractures as it spreads away from the neovolcanic zone⁹ (raising the porosity and lowering the seismic velocity¹⁰) and the cracks are subsequently refilled by hydrothermal alteration products¹¹ (again raising the seismic velocity). The pattern is symmetric, repeating itself to the west of the rise axis, as is seen in the residuals from the rise-axis site (Fig. 2).

Aerial maps of the travel-time residuals and amplitude shadows demonstrate the along-strike continuity of the rise-axis low-velocity zone, and support an assumption of two-dimensional crustal structure. Eighty per cent of the ray paths lie entirely within the crustal domain bounded by the small

overlapping spreading centres¹² at $12^{\circ}37'$ N and $12^{\circ}54'$ N. Residuals and shadows for paths sampling crust beyond this segment are consistent with the pattern within.

Azimuthal anisotropy is clearly part of the signal seen in the residuals, especially at the axis site and the 16-km site. Examination of the range dependence of the anisotropy indicates that it originates in the upper 2 km of crust, and can be treated as a station correction, the size of the correction depending only on the direction to the source. We calculated the best fitting simple anisotropy¹³ ($\cos 2\theta$) at each site, determining a 'fast' direction and magnitude. At all three sites the fast direction is within 10 degrees of the orientation of the rise axis. Assuming the fast direction is parallel to the rise axis, we find an excess residual of ± 0.05 s at the 16-km site—about a 10% difference between the slow and fast velocities. This upper-crustal anisotropy has been attributed to the presence of fluid-filled cracks aligned with the rise axis, although the degree of anisotropy observed here is considerably greater than that observed in much older crust¹⁴. Correcting for anisotropy decreases the variance of the close-range residuals at the 16-km site by $>50\%$. Because estimates of the magnitude of the anisotropy at the other sites are probably contaminated by local structures, which will mimic simple anisotropy, we have corrected the residuals at all sites using the correction from the 16-km site.

By assuming a laterally homogeneous structure, we can map the residuals from the horizontal plane onto a vertical plane normal to the rise axis, placing each residual at the turning point of its ground path. This projection of the anisotropy-corrected data is shown in Fig. 3. The behaviour of the residuals is clearly

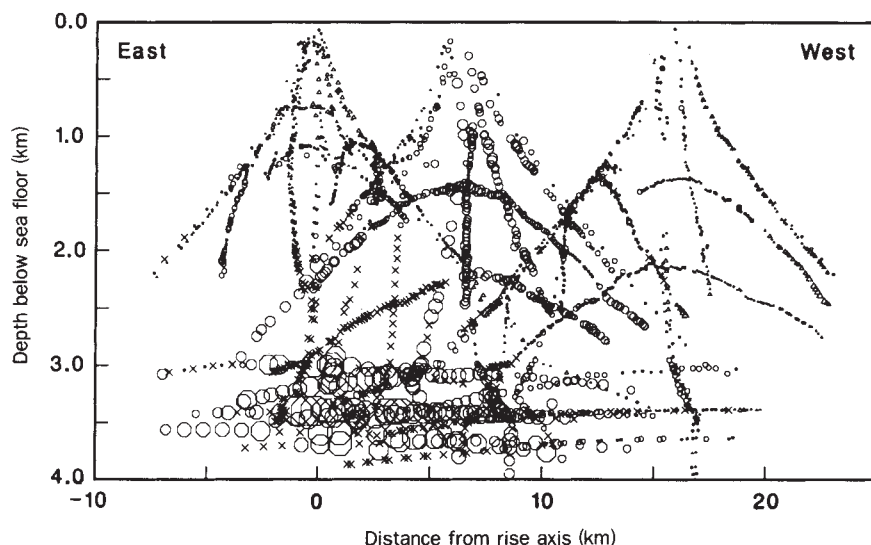


FIG. 3 A cross-sectional view of the residuals, looking along the rise axis. These residuals have been corrected for anisotropy and are plotted at the bottoming points of the ray paths, assuming a laterally homogeneous velocity structure below a flat sea floor. Symbols as in Fig. 2. Note the many large slow residuals and severely attenuated arrivals below the rise crest.

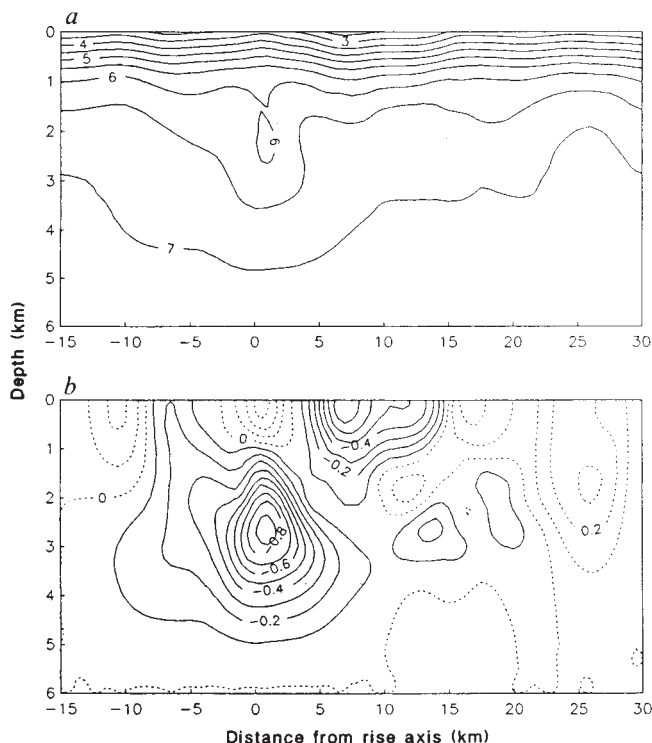


FIG. 4 The final velocity model (a) and the perturbation (b) to the starting model resulting from the tomographic inversion of the residuals in Fig. 3. The structure is poorly constrained below 3.5 km depth and near the sides, and the perturbation is fixed at zero along the sides and bottom of the model. Upper-crustal velocities are fast at the rise axis, then decrease and subsequently increase as the distance from the rise axis increases. The axial low-velocity zone is ~ 6 km wide, measured at the -0.5 km s^{-1} perturbation contour.

dominated by the effects of the axial low-velocity zone; large slow residuals (large circles) and low-amplitude paths (crosses) are concentrated under the rise axis. Ascribing all of the anomaly to the turning point is certainly an oversimplification, however, and tomographic inversion is a superior method for distributing the residuals within the structure. Although three sites would ordinarily be insufficient for a complete three-dimensional inversion, as there would be far too few crossing ray paths, the assumption that the structure is two-dimensional collapses the problem and dramatically improves the apparent resolution.

We have inverted the three-dimensional data set for a smooth two-dimensional crustal structure using 2,314 travel times corrected for bathymetry and anisotropy. To enhance coherence we have used only the source-receiver pairs with ray entry points south of the $12^{\circ}54' \text{ N}$ overlapping spreading centre, a 2-km offset just 7 km north of the axis site. The velocity structure is parameterized as a two-dimensional mesh of 1,162 nodes, defining 2,184 triangles. Each triangle is 1.0 km in width and 0.25 km in height and features a linear velocity gradient; geometrical rays therefore consist of semicircular segments. Rays are traced three-dimensionally through the two-dimensional model, with each triangle acting as an infinite triangular prism. Ray paths, travel times and travel-time partial derivatives are calculated analytically. In the inverse problem we solve for the perturbation to the starting model (the laterally homogeneous model discussed above) which minimizes a weighted sum of the misfit (the residuals in the resulting model) and the roughness of the model perturbation (using a simple difference approximation to the laplacian). The matrix problem is solved using an accelerated 'row-action' method¹⁵, allowing us to take advantage of the sparseness of the matrix. As few of the rays travel below a depth of 3.5 km, or near the sides, resolution is poor in these areas, and the perturbation has been forced to zero along the sides and bottom of the model.

The velocity model resulting from the inversion is shown in Fig. 4. A variance reduction of 87% was achieved in a single iteration, with the new residuals calculated by assuming linearity. Even with this large reduction in the residuals, the new model is quite smooth. Performing the inversion without the anisotropy correction produces models with unreasonably high-amplitude structures, corroborating the existence of sub-

stantial upper-crustal anisotropy. The tomographic inversion also confirms that upper-crustal velocities first decrease and then subsequently increase as the crust ages. The most striking feature in the resulting model, however, is the substantial zone of low velocities beneath the rise axis, indicative of an axial magma chamber. The low-velocity zone outlines a region ~ 6 km wide in which the velocities are depressed by 0.5 – 1.0 km s^{-1} . Although the 'peaked' appearance of the low-velocity zone reflects the amount of smoothing in the inversion, the 6-km width of the -0.5 km s^{-1} contour is relatively stable. The behaviour of the low-velocity zone at depth is not well constrained, and it may extend to the bottom of the model.

The tomographic image of a narrow low-velocity zone is very similar to the results from trial-and-error modelling of the subset of the data⁵ normal to the rise axis. The location of the top of the magma chamber, between 1 and 2 km depth, is consistent with the seismic-reflection observations⁴. Analysis of expanding spread profile data from 13° N on the East Pacific Rise¹⁶ suggests an axial low-velocity zone consisting of hot rock with a small partial melt fraction (velocity reduced 0.5 – 1.0 km s^{-1}) capped by a thin lens with a high partial melt fraction (velocity reduced by over 2.0 km s^{-1}) that gives rise to the strong magma chamber reflector. The travel-time inversion does not resolve the thin lens of very low velocities, but rather the larger, milder low-velocity zone beneath it. $\square$

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New pressure-induced structural transformations in silica obtained by computer simulation

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To reproduce and predict crystal structures from first principles has been a longstanding problem in solid-state physics. We have recently shown¹ that a first-principles many-body calculation for clusters can be used to extract effective pairwise interatomic potentials, which were then used in a molecular dynamics study of the stability of crystalline silica (SiO₂). Here we use this approach in a molecular dynamics study of pressure-induced structural transitions at room temperature for various polymorphs of silica. We predict new structural transitions for low-quartz, low-cristobalite and coesite, in which some of the new phases, appearing without the occurrence of diffusion, comprise mixed arrays of fourfold and sixfold Si-O coordinations. Stishovite, the densest known polymorph of silica, persists up to 250 GPa, with deformation to the CaCl₂ structure. Although a recent theoretical calculation predicts a possible polymorph denser than stishovite, which may be of importance in the Earth's interior², this phase is not obtained by (simulated) compression of stishovite at room temperature, presumably because of the potential barrier between the two structures.

The functional form of the interatomic potentials is expressed as¹

$$U_{ij}(r) = Q_i Q_j / r + f_0(b_i + b_j) \times \exp[(a_i + a_j - r)/(b_i + b_j)] - C_{ij}/r^6$$

where r is the distance between the i th and the j th atoms, $f_0 = 1 \text{ kcal } \text{\AA}^{-1} \text{ mol}^{-1}$, and the potential parameters used here are $Q_O = -1.2$ and $Q_{Si} = +2.4$ in units of elementary charge, $a_O = 2.05$, $a_{Si} = 0.86$, $b_O = 0.176$ and $b_{Si} = 0.033$ in $\text{\AA}$, and $C_{OO} = 4,956$, $C_{OSi} = 1,633$ and $C_{SiSi} = 0$ in $\text{kcal } \text{\AA}^6 \text{ mol}^{-1}$. A drawback with the cluster approach for obtaining pairwise potentials is that, as the approach concentrates on the curvature of potential surfaces, it does not necessarily reproduce the exact enthalpy difference between phases. For example, the observed enthalpy of formation of sixfold-coordinated stishovite³ is $\sim 10 \text{ kcal mol}^{-1}$ higher than that ($-217.8 \text{ kcal mol}^{-1}$) of four-coordinated low-quartz⁴, as compared with the calculated difference of $\sim 1 \text{ kcal mol}^{-1}$. As we are interested in pressure-induced structural transformations, which involve changes of the Si-O coordination number, we slightly modify the potential parameters from those previously obtained¹ to reproduce the enthalpy difference of stishovite and low-quartz. Merely a reduction of the atomic radius a_{Si} by 1% ($0.01 \text{ \AA}$) is sufficient to accomplish this.

Using these potentials, we apply a hydrostatic pressure to each polymorph of silica in the molecular-dynamics study, with both the constant-pressure algorithm of Parrinello and Rahman⁵, and the constant-temperature algorithm of Nosé⁶. The number of atoms in the system is 576 (containing 48 unit cells), 576(64), 768(16) and 576(96) for low-cristobalite, low-quartz, coesite and stishovite, respectively, and we use periodic boundary conditions. The pressure is increased slowly (in steps of 1 GPa, and allowing equilibrium to be attained after each step).

The equations of state obtained for SiO₂ in various polymorphs at room temperature are shown in Fig. 1. The results for low-quartz, coesite and stishovite, before they undergo structural transitions, agree well with the experimental results⁷. The stability of each polymorph is marked. Low-cristobalite shows

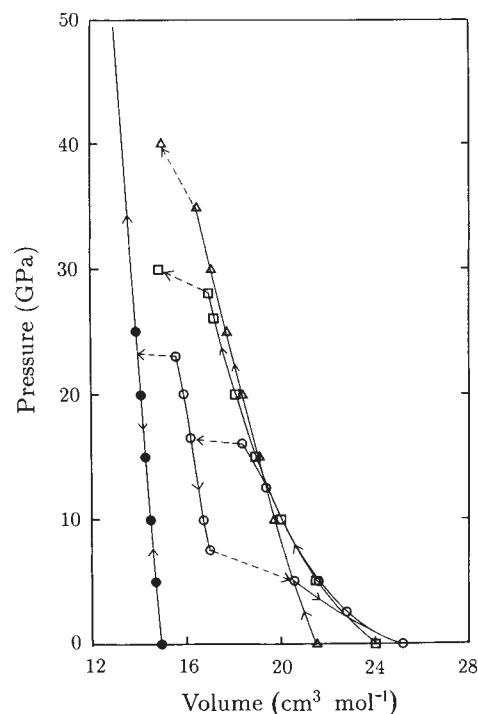


FIG. 1 Pressure-volume relation for low-quartz (□), coesite (Δ), stishovite (●) and low-cristobalite (○) obtained by molecular-dynamics simulation. Solid lines represent continuous change, and broken lines discontinuous change of volume due to a pressure-induced structural transition. Hysteresis is indicated by arrows.

a structural transition, characterized by discontinuous volume reduction, to a novel polymorph at 16.5 GPa, reported here for the first time. The new phase (Fig. 2 and Table 1) has a space group $Cmcm$. The space group of low-cristobalite, $P4_12_12$, has an orthorhombic subgroup $C22_1$, of which $Cmcm$ is another supergroup. The $Cmcm$ phase is stable until the second phase transition, to stishovite, at 23 GPa. The space group of stishovite, $P4_2/mnm$, is in turn a supergroup of $Cmcm$ and $P4_12_12$. Remarkably, this structure includes equal numbers of four- and six-coordinated Si atoms. Note that these structural phase transitions occur without diffusion processes, that is, no disordered states appear at the transition point. With decreasing pressure the $Cmcm$ phase becomes unstable at ~ 7 GPa, resulting in an amorphous phase with structure and density similar to those of low-cristobalite, whereas once stishovite is formed it remains stable against decompression. The reversible transformation between fourfold and sixfold Si-O coordination is consistent with the mechanism suggested by Stolper and Ahrens⁸.

Low-quartz also exhibits a novel structural transition at 28–30 GPa. One of the pressure-induced phases is shown in Fig. 3a. This is basically the α -PbO₂ structure with only sixfold Si-O coordination, which has been proposed⁹, but not established, as a high-pressure form of SiO₂. Another pressure-induced phase was obtained in some molecular-dynamics runs, and is shown in Fig. 3b, c. This phase is also crystalline but contains both four- and six-coordinated Si atoms, as in the $Cmcm$ phase, but in this case the average coordination number is 5.3. Despite their

TABLE 1 Crystallographic data of the $Cmcm$ phase of silica at 15 GPa, 300 K

Orthorhombic cell			
$a = 5.20 \text{ \AA}$; $b = 7.44 \text{ \AA}$; $c = 5.58 \text{ \AA}$			
$\rho = 3.70 \text{ g cm}^{-3}$			
Si (4a)	$x=0$	$y=0$	$z=0$
Si (4c)	$x=0$	$y=0.357$	$z=0.25$
O (8f)	$x=0$	$y=0.223$	$z=0.029$
O (8g)	$x=0.260$	$y=0.481$	$z=0.25$

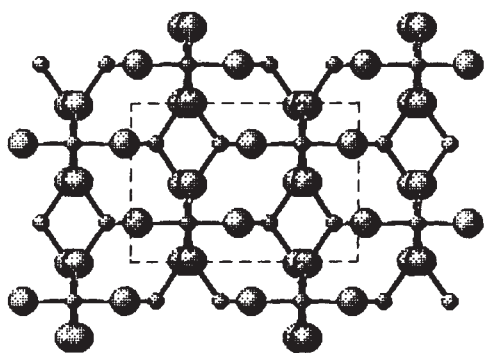


FIG. 2 The new $Cmc2_1$ phase predicted by this study, involving both four- and sixfold coordination, as seen from the a axis, which corresponds to the c axis of low-cristobalite and stishovite. Small spheres represent silicon, and large spheres represent oxygen. The unit cell is shown by dashed lines.

ordered array, however, the unusual, frustrated configuration of the four- and sixfold sites implies considerable distortion of bond lengths and angles. This appears as a random distribution of missing bonds of a finite density, if we regard Si and O as bonded when separated by a distance $<2.16 \text{ \AA}$, a chemically acceptable bound. Peaks in the radial distribution function are also shown to be broadened, as in a glass. The configuration of these missing bonds fluctuates with time (of which Fig. 3 is a snapshot), and they are found to disappear when the pressure is increased to 50 GPa. The direction of the crystal assumes one of the three equivalent directions in the quartz structure, but which of these is selected depends on the run. It is thus conceivable that compression of a macroscopic sample produces domains of these α - PbO_2 or frustrated crystal phases, which may require careful experimental study to distinguish from the conventional amorphous phase. The mixed coordination predicted here could be identified by Raman spectroscopy. The critical

pressures for structural transitions obtained here are consistent with the recent experimental results by Hemley *et al.*⁷ on the amorphization of low-quartz at $\sim 25\text{--}30$ GPa.

These structural transitions of low-quartz are again diffusionless, and the sixfold Si-O coordination returns almost completely to fourfold after decompression to normal pressure. This is consistent with the result of infrared spectroscopy, where the coordination number of amorphous SiO_2 changes reversibly with pressure even at room temperature¹⁰.

Some technical details need to be addressed. The heat of reaction produced during the structural transition is rapidly removed in these molecular-dynamics studies, so that metastable phases are favoured. Also, if the simulation volume contains $3 \times 3 \times 3$ unit cells rather than $4 \times 4 \times 3$, the $Cmc2_1$ phase, which is made of two unit cells of low-cristobalite, is bypassed, and low-cristobalite changes directly into stishovite above 20 GPa. Thus, details of this new polymorph and the associated transition may have to be determined for larger system sizes. But we expect that the same kind of transition, involving mixed coordination numbers, will occur in real systems. An experimental study is in progress¹¹.

For coesite, the basic cell changes slightly from monoclinic to triclinic at 15–20 GPa, which may be associated with the observed change in the Raman spectrum at 22–25 GPa (ref. 12). Coesite also undergoes a structural transition into a sixfold-rich phase at 35–40 GPa, which is consistent with the experimentally reported amorphization at 30–34 GPa (ref. 7).

Finally, stishovite exhibits no drastic structural change up to 250 GPa, the highest pressure studied here, except that the structure eventually becomes orthorhombic (with the $CaCl_2$ structure). This result suggests that the recently proposed $Pa\bar{3}$ phase², predicted to be denser and thermodynamically more stable than stishovite above 60 GPa, is difficult to attain dynamically by simple compression at room temperature¹¹. This does not necessarily imply, however, that the $Pa\bar{3}$ phase is unstable. Even if the $Pa\bar{3}$ phase is more stable than stishovite, it is possible

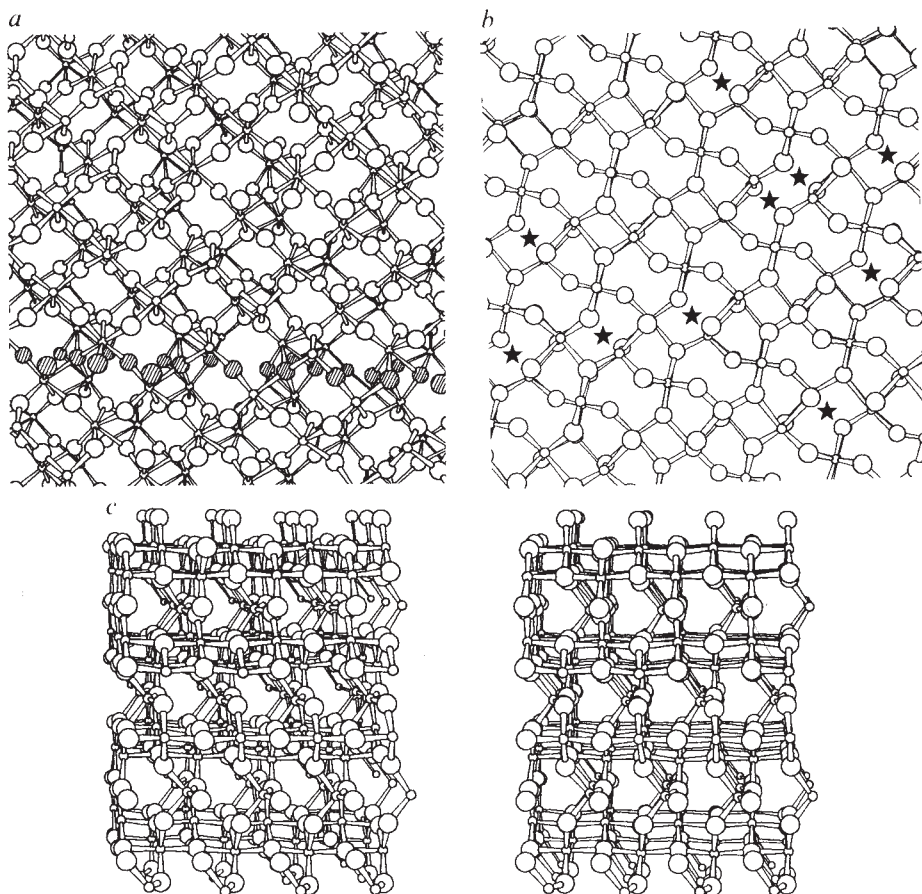


FIG. 3 SiO_2 at ~ 30 GPa obtained by compression of low-quartz at room temperature: *a*, The α - PbO_2 phase with some defects. *b*, *c*, A frustrated crystal phase, where *b* is a projection to the plane normal to the a axis and *c* is a stereoscopic view seen from the c axis of starting low-quartz. Small spheres represent silicon and large spheres represent oxygen. A plane of close-packed oxygen atoms in the α - PbO_2 phase is shown by hatching in *a*, and missing bonds of the frustrated crystal phase are marked with $\star$ in *b*.

that the potential barrier separating the two phases in the atomic configuration space would be higher than kT at room temperature so that a diffusion process at high temperatures would be necessary for the transition. This problem could be tackled by the molecular-dynamics method, which is also applicable to transitions involving diffusion processes.

The potentials considered here, which have been shown to reproduce (within 10%) the structures and bulk moduli of virtually all the known polymorphs of silica at room temperature and normal pressure, have proved to be adequate for simulation of such dynamical processes as structural transitions. These results suggest that the application of high pressure at room temperature may provide routes to new polymorphs. □

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Evidence from mitochondrial DNA that African honey bees spread as continuous maternal lineages

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AFRICAN honey bees have populated much of South and Central America and will soon enter the United States. The mechanism by which they have spread is controversial. Africanization may be largely the result of paternal gene flow into extant European populations or, alternatively, of maternal migration of feral swarms that have maintained an African genetic integrity. We have been using both mitochondrial and nuclear DNA restriction fragment length polymorphisms to follow the population dynamics between European and African bees. In earlier reports, we suggested that if African honey bees had distinctive mitochondrial (mt) DNA, then it could potentially distinguish the relative contributions of swarming and mating to the Africanization process^{1,2}. Because mtDNA is maternally inherited, it would not be transmitted by mating drones and only transported by queens accompanying swarms. Furthermore, the presence of African mtDNA would reflect unbroken maternal lineages from the original bees introduced from Africa. The value of mtDNA for population studies in general has been reviewed recently^{3,4}. Here we report that 19 feral swarms, randomly caught in Mexico, all carried African mtDNA. Thus, the migrating force of the African honey bee in the American tropics consists of continuous African maternal lineages spreading as swarms. The mating of African drones to European queens seems to contribute little to African bee migration.

Thirty years ago African honey bees (*Apis mellifera scutellata*⁵) were released in Brazil. Since that time they have spread

throughout most of South and Central America^{6–9}. They are now moving through Central Mexico at an unabated pace of more than 300 miles per year. The migrating front will reach Texas in mid-1990. In subsequent years, the bees are expected to populate the southern tier of the United States⁹. Extremely defensive stinging and other characteristics make African bees difficult to manage. Many beekeepers will go out of business, thus reducing the availability of honey bees for commercial pollination^{9,10}. The threat the bees pose to the public has been sensationalized but is, nevertheless, real¹¹.

Apart from the agricultural, economic and public health concerns, migration of the African honey bee in the Americas is an amazing population biology phenomenon. During the course of their spread, the tropically adapted African bees have established a substantial feral population. They have largely replaced the more poorly adapted European subspecies whose presence in the tropics has been mostly limited to managed apiaries^{8,12,13}. The mechanisms responsible for the spread of African bees and elimination of European bees are not well documented and have become a controversial subject. Some reports contend that genetic displacement, that is gene flow through the flight of African drones mating with extant European queens, is the primary driving force behind the Africanization process^{14,15}. Enhanced production and survival of African drones has been postulated to drive introgression in their favour^{16,17}. The very term 'Africanized bees', commonly accepted to describe African-derived populations in South and Central America, implies that the bees are mostly European with introgression of African genes. This was a reasonable assumption at the time African bees were introduced, when they were reported to be a small minority compared with the European population⁶. Apiaries do progressively exhibit African behavioural^{7,8,18}, genetic (allozyme

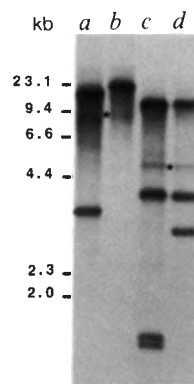


FIG. 1 Restriction fragment length polymorphisms between European and African mtDNA. *Bgl*II cuts European mtDNA (a) into two fragments, 13.2 kilobase (kb) and 3.8 kb²³, and African mtDNA (b) once to form a single 17-kb fragment. *Eco*RI digests European mtDNA (c) to generate four fragments, 10.0 kb, 3.8 kb, 1.8 kb and 1.4 kb, and African mtDNA (d) to form three fragments, 10.0 kb, 3.8 kb, and 3.2 kb. The African *Eco*RI pattern, previously reported in Brazilian bees, results from a site loss between the 1.8 kb and 1.4 kb European fragments²².

METHODS. Mitochondrial DNA was isolated³⁶ from fresh 4- to 5-day-old larvae from a colony in Florida, and the isolate served as a radioactive probe to detect mtDNA fragments in blots. As a probe, the mtDNA was labelled by nick translation with [³²P] deoxycytidine or end-labelled after *Eco*RI digestion³⁷. The rationale behind using mtDNA as a probe, rather than obtaining pure mtDNA from each sample, was to allow analysis of samples preserved in alcohol and shipped at ambient temperatures. It was also to establish an approach more amenable to routine testing in the future. Contaminating nuclear DNA was present in sufficient amounts in the probe preparations to detect chromosomal DNA as background or as bands (asterisks), particularly in samples preserved in alcohol. The mitochondrial bands were, nevertheless, clearly distinguishable from the nuclear DNA. Positions and sizes (in kilobases) of Lambda bacteriophage *Hind*III fragments are indicated as markers.

frequencies)¹⁹, and subtle morphological characteristics²⁰, as a result of gene flow from African drones. It has not been demonstrated, however, that these hybrid bees constitute or contribute significantly to the migrating 'Africanizing' force, or even persist in established African bee populations. Alternatively, the 'Africanizing' force may consist almost exclusively of swarms from feral African bee populations that have largely retained their genetic integrity^{9,13}. African honey bees are notorious for their propensity to swarm^{7-9,21}, which could logically account for their rapid spread. Swarming is the mechanism of maternal migration, whereby the queen and about half the workers leave their hive and fly to another location to establish a new colony.

Restriction endonucleases *EcoRI* and *BglII* each generate fragment patterns that distinguish European from African mtDNA (Fig. 1)^{22,23}. The European patterns were seen in a broad sampling of honey bee stocks from across the United States, maintained as a closed population in Tucson, Arizona (USDA-ARS)²⁴ (total of 20 colonies tested, of which 5 are shown in Fig. 2 (group A)). The African patterns were obtained from colonies located in the vicinity of Pretoria, South Africa (total of 10 colonies tested, of which 5 are shown in Fig. 2 (group E)). The African bees introduced to South America came from this region⁶. In January 1988, near Tapachula, Mexico, we collected samples from six colonies established before African

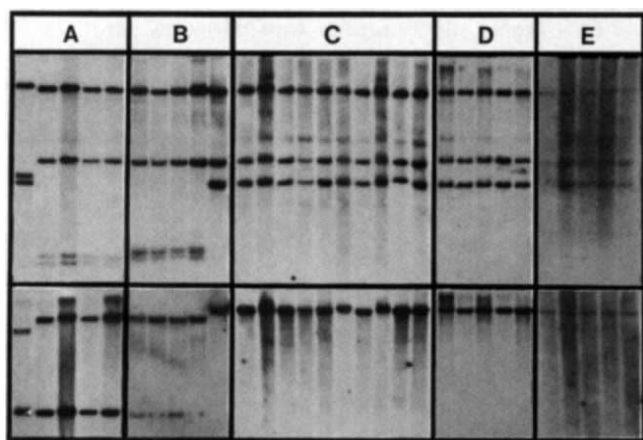


FIG. 2 Samples of honey bee DNA digested with *EcoRI* (upper panels) and *BglII* (lower panels), separated by electrophoresis, blotted and hybridized to radioactive mtDNA. Each lane represents a single colony, a mixture of progeny from a single queen: Group A, samples from a closed population, maintained by the USDA-ARS in Tucson, of European bee stocks from across the United States. The sample in the first lane exhibits a less common *EcoRI* pattern consisting of three fragments of approximate sizes 11.0 kb, 3.4 kb, and 3.2 kb. All United States bees tested so far carry one of the two mtDNA patterns which appear to be characteristic of specific European races²⁵; group B, mtDNA from an apiary established in Mexico before African bee arrival. Four have European mtDNA, whereas the sample in the last lane exhibits African mtDNA. The latter suggests a possible direct African queen takeover; group C, mtDNA from feral Mexican swarms; group D, from Venezuelan managed colonies; group E, from the Republic of South Africa. All carry the *EcoRI* and *BglII* African mtDNA patterns.

METHODS. Before analysis, most of the United States and Venezuelan samples were frozen as fresh material. The Mexican samples were equilibrated in cold 95% alcohol before being transported on ice (4 °C). Samples from South Africa were also equilibrated in alcohol but were mailed at ambient temperatures. The transit time of more than 2 weeks apparently caused some loss of mtDNA, but the fragments are visible despite the high background. All samples were either pupae or larvae. The use of brood ensures their colony origin, as adults can drift among hives. Furthermore, the soft brood tissue facilitates DNA isolation. Samples in alcohol were allowed to equilibrate in 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM MgCl₂ overnight at 4 °C with gentle agitation. A protocol was used to obtain an isolate from each sample enriched for mtDNA³⁷. Endonuclease digestion, electrophoretic separation and blotting onto nylon membranes followed standard procedures³⁸, with modifications previously described⁴.

bee arrival fifteen months earlier. Five carried European mtDNA fragment patterns and one carried the African patterns (5 shown; Fig. 2 (group B)). Samples were also collected from thirteen feral swarms that had occupied bait hives maintained by the Mexican agency SARH (Secretary of Agriculture and Hydrologic Resources). In July 1988, we sampled six additional swarms about 400 km further northwest, in the vicinity of Las Choapas and San Andrés Tuxtla. All nineteen feral swarms had African mtDNA (10 shown; Fig. 2 (group C)). Bees from managed Venezuelan colonies (total of 10 colonies of which 5 are shown, in Fig. 2 (group D)) and from Costa Rica (total of 3, not shown) also carried African mtDNA. Almost identical results were obtained independently and are described in the accompanying paper²⁵.

Most of the established Mexican colonies, seen here to have European mtDNA, exhibited low levels of European nuclear DNA alleles²⁶. This provides further evidence that colonies do become 'Africanized' as a result of gene flow from African drones. However, observations of managed apiaries, rather than of feral populations, would give an exaggerated impression of the importance of paternal introgression¹³. Since the African bee introduction in 1956, there have been, as a very rough estimate, 150 generations that together have migrated a total of more than five thousand miles to reach Mexico. For the swarms to carry African mtDNA, not a single intervening generation could have been the result of an African drone mating to a European queen. Such matings certainly occur after African swarms enter an area, and the progeny can be correctly called 'Africanized bees'. Nevertheless, despite an African nuclear composition inherited from drones, European mother lines do not contribute significantly, if at all, to the African bee migrating force.

Judging from the absence of European mtDNA in managed Venezuelan colonies, 'Africanized bees' may contribute little to established African bee populations as well. Direct takeover of colonies by invading African swarms is believed to have a minor role in the Africanization of apiaries^{8,9,27}, but, cumulatively over time, it could account for a significant loss of European maternal lines. The apiary colony in Mexico found to have African mtDNA suggests a take-over, although we cannot ascertain, after the fact, that the bees did not simply colonize an empty hive. Unless actively maintained, European maternal lines in the tropics may disappear eventually through attrition. If introgression of African nuclear genes into European colonies has a role in Africanization, it would be to increase gene frequencies and, thus, by back-crossing, increase the homozygosity of the surviving African maternal lines.

Some minor 'Europeanization' of feral African bees seems to occur as they migrate into regions occupied by European apiaries. In the feral Mexican swarms, European nuclear DNA alleles were absent or present at low levels, in most cases, too low for the queens to be heterozygous²⁶. Hence, the European markers did not reflect ancestral hybridization with European bees and would have come from a minority of drones in just the previous matings with the queens. It remains to be determined how long such European alleles persist in African populations. The markers are almost completely absent from bees sampled from Venezuela^{2,26}, suggesting a loss over time. A temporal transition towards a more African morphology, in the years following passage of the migrating front, has been reported¹². This may reflect an overwhelming increase in African gene frequencies as the feral population becomes established^{9,12,13}. A number of mechanisms could select against European-African hybrids, which would affect a small percentage of bees in African feral populations but a large proportion in 'Africanized' apiaries.

Mitochondrial DNA is capable of introgressing, in some cases, even across species boundaries²⁸⁻³⁰. Such introgression would be prevented either by reproductive isolation, non-viable hybrids, or non-neutral mtDNA^{28,29}. Of the three, only the latter could explain the failure of 'Africanized' European maternal

lines to enter the migrating feral population. The migrating front, by its very nature, would represent the furthest flying African bees. African bees fly much further than European bees³¹. It is tempting to speculate that European mitochondria may limit a metabolic capability needed for long distance dispersal. The African bee's shorter developmental period and lifespan suggest metabolic differences^{8,21,32}. Interspecies incompatibility is sometimes manifested as impaired mitochondrial function^{4,33-35}. It is conceivable that interactions between mitochondrial and nuclear factors, from widely divergent subspecies, could be suboptimal and thus contribute a disadvantage to hybrids. The dynamics of African honey bee nuclear and mitochondrial gene spread will probably be quite different in the temperate United States and may point to underlying selective processes. □

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Neotropical Africanized honey bees have African mitochondrial DNA

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NON-INDIGENOUS African honey bees have invaded most of South and Central America in just over 30 years¹. The genetic composition of this population and the means by which it rapidly colonizes new territory remain controversial. In particular, it has been unclear whether this 'Africanized' population has resulted from interbreeding between African and domestic European bees, or is an essentially pure African population. Also, it has not been known whether this population expanded primarily by female or by male migration. Restriction site mapping of 62 mitochondrial DNAs of African bees from Brazil, Venezuela and Mexico reveals that 97% were of African (*Apis mellifera scutellata*) type. Although neotropical European apiary populations are rapidly Africanized by mating with neotropical African males, there is little reciprocal gene flow to the neotropical African population through European females. These are the first genetic data to indicate that the neotropical African population could be expanding its range by female migration.

New World domesticated honey bees are descendants of introduced Old World subspecies², primarily *A. m. mellifera*, *ligustica* and *carnica*³⁻⁵. In 1956, 47 queens of African *A. m. scutellata* were imported from South Africa to Brazil as part of a breeding programme to develop hybrid strains better suited to the South American climate. Twenty-six queens escaped and

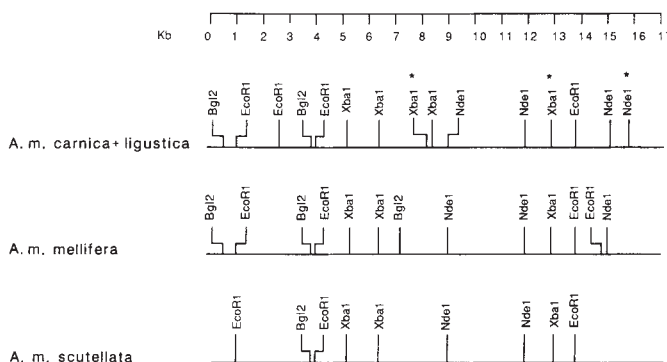


FIG. 1 Maps of *XbaI*, *EcoRI*, *BglII* and *NdeI* cleavage sites in mtDNA of European and African honey bee subspecies. The restriction fragments generated by each of these enzymes distinguish African *A. m. scutellata* from European *A. m. carnica*, *ligustica* and *mellifera*. The *XbaI* fragment from approximately 5.2-6.4 (kilobases) (kb) is 80-250 base pairs larger in *A. m. mellifera* than in *A. m. scutellata*. *XbaI* and *EcoRI* were selected for use in a survey of neotropical African bees because they produce multiple restriction fragments in the mtDNA of each subspecies and the fragments they generate end-label reliably. MtDNA was prepared^{27,28} and digested with the restriction enzymes *AccI*, *AvaI*, *BclI*, *BglII*, *EcoO109*, *EcoRI*, *EcoRV*, *HincII*, *HindIII*, *NdeI*, *PstI*, *PvuII*, *SpeI*, *XbaI* and *XhoI*, generating a total of 40 cleavage sites (29-33 per subspecies) which were mapped by means of double digests²⁷⁻²⁹. No consistent differences were found between the mtDNA restriction maps of *carnica* and *ligustica*. Per cent sequence divergences among mitochondrial genotypes were calculated³³ from non-degenerate recognition sites. Per cent sequence divergences (standard deviations) within populations are *A. m. carnica/ligustica*: 0.33% (0.33) to 0.97% (0.58); *A. m. mellifera*: 0.34% (0.34) to 0.71% (0.51); *A. m. scutellata*: 0.78% (0.56). Per cent sequence divergences between subspecies are *A. m. carnica/ligustica* versus *A. m. mellifera*: 2.91% (1.12) to 4.05% (1.34); *A. m. carnica/ligustica* versus *A. m. scutellata*: 2.23% (0.96) versus 3.04% (1.16); *A. m. mellifera* versus *A. m. scutellata*: 2.3% (1.01) to 3.44% (1.01). Restriction maps are based on 17 hives of *A. m. carnica* from West Germany, Austria and Yugoslavia; nine hives of *A. m. ligustica* from Italy; 22 hives of *A. m. mellifera* from Norway, Sweden, Denmark and France; and 19 hives of *A. m. scutellata* from South Africa.

established a feral neotropical African population^{6,7}. These bees subsequently spread across South and Central America at the rate of as much as 500 km per year^{1,8}, and are expected to arrive in the United States in 1989–90 (ref. 1). Their habits of frequent swarming and absconding make them undesirable for commercial bee keeping, and their aggressive nest defence, which has resulted in numerous human fatalities, is viewed as a serious threat to public health^{1,9–12}.

Gene flow from feral neotropical African bees to managed European apiaries is well documented. Feral neotropical African bees attain high population densities within 2–3 years after arrival in a region, followed by Africanization of local apiaries^{12,13}. Africanization entails appearance of behavioural^{11,13–15} and morphological¹⁶ characteristics typical of *A. m. scutellata*, as well as changes in allozymes^{17,18} and nuclear DNA^{19,20}. Because neotropical African colonies produce more drones than European colonies, and in some circumstances suppress production of drones by European colonies²¹, Africanization of apiaries occurs primarily through mating of domestic European queens with abundant neotropical African drones^{21,22}, and less commonly by take-over of hives by African swarms^{13,21}.

Because young queens return to their colonies after mating flights, the rapid spread of neotropical African bees is often attributed to the dispersal of drones, which may fly several kilometres from their colonies to join mating congregations. But queens do disperse later in their life-cycle. After producing a daughter queen, who will inherit the nest, the older queen plus a retinue of workers swarm, leaving the old nest and establishing a new colony elsewhere. African and European/African hybrid queens swarm more frequently^{8,23–25} and travel further^{8,26} than typical European queens. Thus, whenever apiaries become Africanized, large numbers of queens carrying European mitochondrial (mt) DNA enter the feral population.

A key question is the fate of these swarms. If they survive in the feral population, the Africanized apiaries become sources of swarms which contribute European mtDNA to the feral neotropical African population. Alternatively, if few of these swarms survive in the feral population, then little European mtDNA will be introduced to the African population.

We tested these hypotheses by examination of maternally inherited mitochondrial genomes in the neotropical African

population. We constructed mtDNA restriction enzyme cleavage maps for the European subspecies *A. m. ligustica*²⁷, *A. m. carnica*^{27,28} (which had similar restriction maps), and *A. m. mellifera*^{27,28}, and for the African subspecies *A. m. scutellata*^{27,29} (Fig. 1). The restriction enzymes *EcoRI* and *XbaI* were selected for a survey of neotropical African populations because each produces unique, multi-fragment length polymorphisms in the mtDNAs of *A. m. mellifera*, *scutellata* and *carnica/ligustica* (Figs 1, 2).

MtDNA was prepared (Fig. 3) from ten neotropical African hives from Ribeirão Preto, Brazil (near the point of escape of *A. m. scutellata*); twelve hives from Acarigua, Venezuela; 39 feral swarms from Tapachula, Chiapas, Mexico, (near the current front of neotropical African bee expansion); and from five hives from Ribeirão Preto, Brazil (near the point of escape of with European queens. Samples were digested with *XbaI* and *EcoRI* and the resulting restriction fragments compared with those of European and African subspecies. Nine of ten Brazilian, all twelve Venezuelan, and 38 of 39 Mexican mtDNAs were of African (*scutellata*) type (Fig. 3). Only one Brazilian and one feral Mexican mtDNA were of European (*carnica* or *ligustica*) type. In the apiary sample, four hives had European mtDNA and one hive had African mtDNA, indicating a colony take-over by an African queen.

Thus, the feral neotropical African population does not include a high frequency of swarms with European mtDNA, showing that there is little gene flow from European queens into this population. Hall and Muralidharan (see accompanying paper) arrive at the same conclusions in their study of North American and neotropical African honey bee mtDNA.

These data show that the mitochondrial genomes of a small number of African females have rapidly colonized most of a

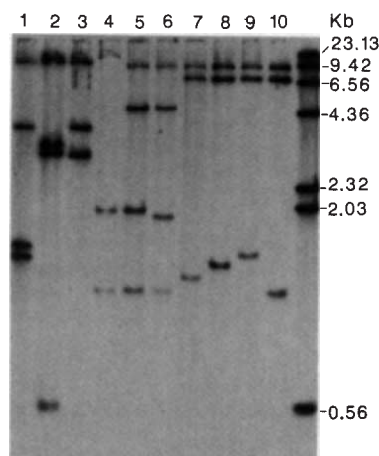


FIG. 2 Autoradiograph of 1% agarose gel showing honey bee mtDNA digested with *EcoRI* (lanes 1–3) and *XbaI* (lanes 4–10). The *EcoRI* restriction fragment pattern in lane 1 is found in *A. m. carnica* and *ligustica*; the pattern in lane 2 is found in *A. m. mellifera*; the pattern in lane 3 is found in *A. m. scutellata*. The *XbaI* restriction fragment patterns in lanes 4–6 are found in *A. m. carnica* and *ligustica*; the pattern and size variants in lanes 7–9 are found in *A. m. mellifera*; the pattern in lane 10 is found in *A. m. scutellata*. The last lane is a size standard (*HindIII*-digested lambda phage DNA). MtDNA samples were prepared, digested and end-labelled as described^{27–29}.

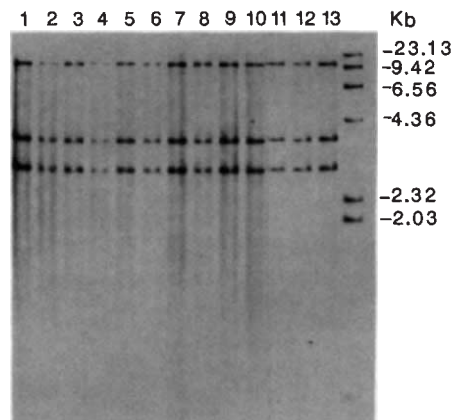


FIG. 3 Autoradiograph of 1% agarose gel showing honey bee mtDNA digested with *EcoRI*. Lanes 1–9, nine Mexican Africanized swarms; lanes 10–11, two Venezuelan Africanized swarms; lane 12, a Brazilian Africanized hive; lanes 13–14, two South African *A. m. scutellata* hives. The last lane is a size standard (see Fig. 2). Mexican samples were collected in January 1988; Venezuelan samples in 1987; Brazilian and South African samples in 1986.

METHODS. MtDNA for population surveys was prepared as follows: 15 thoraces from adult worker hive mates were powdered in liquid nitrogen, suspended in 10 ml of 10 mM Tris, 10 mM NaCl, 200 mM EDTA, pH 7.7, and ground for 30 s with a Tekmar Tissumizer; 1.3 ml 20% sodium dodecyl sulphate was added and the mixture incubated at 23 °C for 15 min; 2 ml of CsCl-saturated 10 mM Tris, 10 mM EDTA, pH 7.7, was added and the mixture incubated on ice for 15 min; cellular debris and precipitated SDS were removed from the supernatant by centrifugation for 10 min at 17,000 g, 4 °C; 0.5 ml of 2 mg ml⁻¹ propidium iodide was added to the supernatant and the density adjusted to 1.52–1.53 g ml⁻¹ with solid CsCl. Samples were loaded into a Beckman Vti 65.2 vertical rotor and centrifuged for 10–15 hours at 55,000 r.p.m.. Sample collection and analysis have been described elsewhere^{27–29}.

TABLE 1 Mean allele frequencies for Mdh¹⁰⁰ and Hk¹⁰⁰ in five honey bee populations

Population	f (Mdh ¹⁰⁰)	N	f (Hk ¹⁰⁰)	N
European subspecies	0.18 (a)	20	1.00 (d)	27
New World Europeans	0.23 (a)	84	0.95 (d)	40
Neotropical Africans				
Mexican	0.70 (b)	56	0.63 (e)	56
non-Mexican	0.92 (c)	21	0.45 (f)	191
African <i>A. m. scutellata</i>	0.99 (c)	25	0.29 (f)	15

Mean allele frequencies (f) for Mdh¹⁰⁰ and Hk¹⁰⁰ in five honey bee populations, weighted by number of hives sampled (N). Frequencies of Mdh¹⁰⁰ and Hk¹⁰⁰ are significantly independent among populations (Mdh: G, the likelihood ratio chi-square, =137.21; Hk: G=146.6; 4 d.f., 1 *P*<0.001); frequencies with the same letter (a–f) are not significantly different (*P*>0.05, simultaneous procedure for test of independence³⁴). Frequencies presented include original and previously published data. New data: Mdh in European *A. m. mellifera* and *A. m. carnica* (collected 1987 by O.R.T. and D.R.S.); Mdh and Hk in European populations from Costa Rica (1986), in neotropical African populations from Mexico (1988) and in South African *A. m. scutellata* (1986). Previously published data: Mdh in *A. m. ligustica*³⁵, New World Europeans from the United States³⁶ and Brazil³⁰, neotropical Africans from Brazil¹⁷, and *A. m. scutellata*¹⁷; Hk in European *A. m. ligustica* and *A. m. carnica*³² and New World Europeans from the United States and Mexico³², Mdh and Hk in New World Europeans from the United States³¹ and neotropical African populations from Costa Rica³¹. Samples from Africanized apiaries were avoided where possible. There is no significant difference in allele frequencies between European subspecies and New World Europeans, nor between long-established (non-Mexican) neotropical African populations and *A. m. scutellata*. There are significant differences between Mexican and long-established (non-Mexican) populations of neotropical African bees. Although there may be gene flow from the European to the African population (through drones) when the African population initially invades new territory, allele frequencies in long-established neotropical African populations are not different from those of South African *A. m. scutellata*.

continent. In addition, allele frequencies of biparentally inherited allozymes (malate dehydrogenase (Mdh), and hexokinase (Hk) distinguish African *A. m. scutellata* from European subspecies^{18,30–32} and do not differ significantly between South African *A. m. scutellata* and long-established neotropical African populations (Table 1). Thus, an essentially African population is expanding in neotropical habitats through migration and colonization of new territory by African females. The paucity of European mtDNA in the feral neotropical African populations may be due to selection against some trait(s) of European or European/African hybrids in tropical habitats, to competitive interactions between European and African bees or to other factors.

The invasion of the New World by African bees provides an unusually clear example of asymmetrical gene flow between two conspecific populations. There is no evidence that the feral neotropical African population has become extensively 'Europeanized'. Future research into the management of neotropical African honey bees should emphasize the factors that limit gene flow from European to African populations and the potential competitive interactions between neotropical African bees and the large North American feral European population. □

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Associative long-term depression in the hippocampus induced by hebbian covariance

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A BRIEF, high-frequency activation of excitatory synapses in the hippocampus produces a long-lasting increase in synaptic strengths called long-term potentiation (LTP)¹. A test input, which by itself does not have a long-lasting effect on synaptic strengths, can be potentiated through association when it is activated at the same time as a separate conditioning input^{2–4}. Neural network modelling studies have also predicted that synaptic strengths should be weakened when test and conditioning inputs are anti-correlated^{5–8}. Evidence for such heterosynaptic depression in the hippocampus has been found for inputs that are inactive^{2,9} or weakly active³ during the stimulation of a conditioning input, but this depression does not depend on any pattern of test input activity and does not seem to last as long as LTP. We report here an associative long-term depression (LTD) in field CA1 that is produced when a low-frequency test input is negatively correlated in time with a high-frequency conditioning input. LTD of synaptic strength is also produced by activating presynaptic terminals while a postsynaptic neuron is hyperpolarized. This confirms theoretical predictions⁸ that the mechanism for associative LTD is homosynaptic and follows a hebbian covariance rule⁷.

We searched for conditions under which the stimulation of a hippocampal pathway, rather than its inactivity, could produce, depending on the pattern of stimulation, either long-term

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depression or potentiation of synaptic strengths. The conditioning stimulus (Fig. 1), based on the finding that bursts of stimuli at 5 Hz are optimal in eliciting LTP¹⁰, was applied to Schaffer collateral/commissural fibres; the test stimulus was applied to a subicular input on the opposite side of the recording site. Each shock of the test input was either superimposed on the middle of each burst of the conditioning input (in phase) or occurred symmetrically between the bursts (out of phase).

Extracellular, evoked-field potentials were recorded from the apical dendritic and somatic layers of CA1 pyramidal cells. The low-frequency train of test stimuli was first applied alone and did not itself induce long-lasting changes. The conditioning site was then stimulated alone, which elicited homosynaptic LTP of the conditioning pathway but did not significantly alter amplitude of responses to the test input. When test and conditioning inputs were activated in phase, there was an associative potentiation of the test input synapses (Fig. 2a). Both the population excitatory postsynaptic potential (e.p.s.p.) and population action potential were significantly enhanced.

By contrast, when test and conditioning inputs were applied out of phase, we observed an associative long-term depression

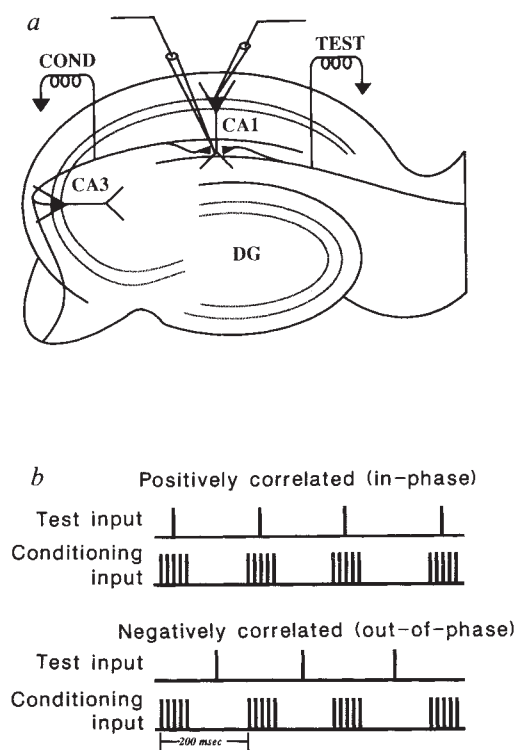


FIG. 1 Hippocampal slice preparation and stimulus paradigms. *a*, Schematic diagram of the hippocampal slice *in vitro* showing recording sites in the CA1 pyramidal cell somatic and dendritic layers, and stimulus sites activating Schaffer and commissural collaterals (COND) and subicular afferents (TEST). *b*, Schematic diagram of stimulus paradigms used. Conditioning input stimuli, four trains of 100-Hz bursts. Each burst had 5 stimuli and the interburst interval was 200 msec. Each train lasted 2 seconds and had a total of 50 stimuli. Test input stimuli, four trains of shocks at 5-Hz frequency, each train lasting for 2 seconds. When these inputs were in-phase, the test single shocks were superimposed on the middle of each burst of the conditioning input, as shown. When the test input was out of phase, the single shocks were placed symmetrically between the bursts.

METHODS. Hippocampal slices (400 μ m thick) were prepared by standard methods¹⁴ and incubated in an interface slice chamber at 34–35 °C. Extracellular recording electrodes were filled with 2 M NaCl and had 1–5 M Ω resistance. Intracellular micropipettes were filled with 2 M potassium acetate and had 70–120 M Ω resistance. Tips of bipolar glass-insulated, platinum wire, stimulating electrodes were 50- μ m in diameter; all electrodes were prepared by standard methods¹⁴.

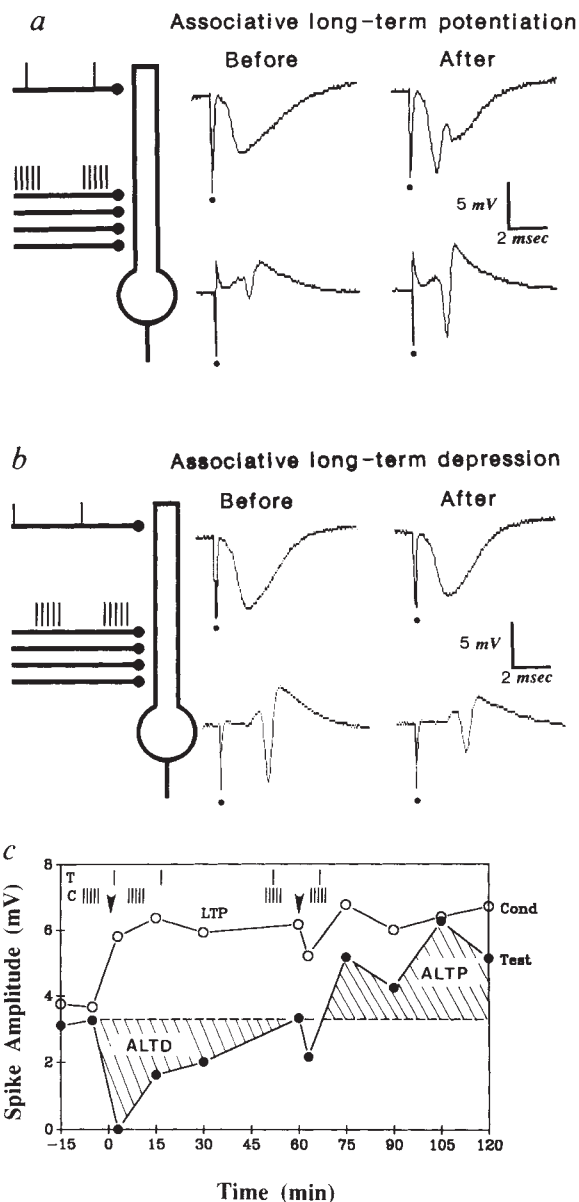


FIG. 2 Illustration of associative long-term potentiation (LTP) and associative long-term depression (LTD) using extracellular recordings. *a*, Associative LTP of evoked e.p.s.p.s and response of the population action potential in the test input. Test responses are shown before and 30 min after application of test stimuli in phase with the coactive conditioning input. *b*, Associative LTD of evoked e.p.s.p.s and responses of population spikes in the test input. Test responses are shown before and 30 min after application of test stimuli out of phase with the coactive conditioning input. *c*, Time course of the changes in population spike amplitudes for a typical experiment. Inset (top) shows the stimulus patterns for the test (T) and conditioning (C) inputs, and arrows show the time of stimulation. Single responses from the conditioning input (open circles), show that the high-frequency bursts (5 pulses of frequency 100 Hz at 200-msec intervals; see Fig. 1) elicited synapse-specific LTP, independent of other input activity. Single responses from the test input (filled circles) show that stimulation of the test pathway, out of phase with the conditioning one, produced associative LTD (ALTD) of this input. In phase stimulation of the same pathway elicited associative LTP (ALTP). In 20 slices the average change in the maximum initial slope of the population e.p.s.p. was $+49.8 \pm 7.8\%$, and the average increase of the population action potential amplitude in 14 slices was $+65.4 \pm 16.0\%$. (Results given as mean percent change $\pm$ s.e.m.) The average reduction of the population spike during associative LTD in 10 slices was $-46.5 \pm 11.4\%$, with a smaller decrease in the average maximum initial slope of the population e.p.s.p. of $-13.8 \pm 3.5\%$ in 13 slices. The duration of associative LTD was at least 30 min and up to 3 h following stimulation. The amplitude and duration of associative LTD or LTP could be increased by stimulating input pathways with more trains of shocks.

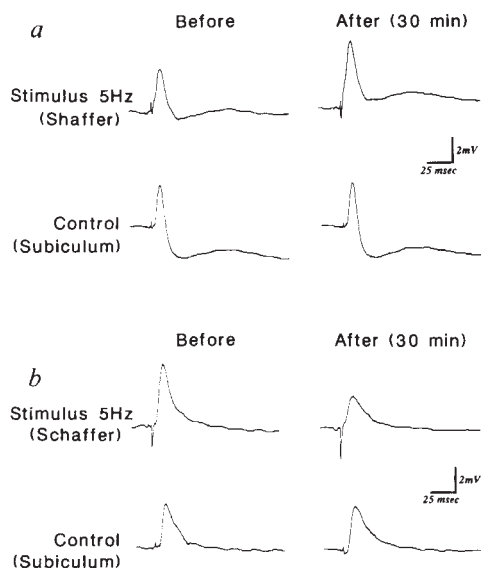


FIG. 3 Pairing of postsynaptic hyperpolarization with stimulation of synapses on CA1 hippocampal pyramidal neurones produces LTD specific to the activated pathway, while pairing of postsynaptic depolarization with synaptic stimulation produces synapse-specific LTP. *a*, Intracellular evoked e.p.s.ps are shown at stimulated (Stimulus 5 Hz, Schaffer) and unstimulated (Control, Subiculum) pathway synapses before and 30 min after pairing depolarizing current injection with 5-Hz synaptic stimulation (the constant +2.0 nA current produced a 20-mV depolarization of the soma without synaptic stimulation). The stimulated pathway exhibited associative LTP of the e.p.s.p., whereas the control, unstimulated input showed no change in synaptic strength. (RMP = -65 mV; R_N = 35 M Ω). *b*, Intracellular e.p.s.ps evoked at stimulated and control pathway synapses before and 30 min after pairing a 20-mV hyperpolarization at the cell soma with 5-Hz synaptic stimulation (the constant -1.0 nA current produced a 20-mV hyperpolarization of the soma in the absence of synaptic stimulation). The input (Stimulus 5 Hz), activated during the hyperpolarization, showed associative LTD of synaptic evoked e.p.s.ps, while synaptic strength of the silent input (control) was unaltered (RMP = -62 mV; R_N = 38 M Ω). The cell fired action potentials during the depolarizing current injection, but not during injection of the hyperpolarizing current. In five cells, a depolarizing current injection paired with the test input led to an average increase of $+110 \pm 31.3\%$, while a control input inactive during the stimulation only changed by $+15.5 \pm 5.4\%$. In five different cells, prolonged hyperpolarizing current injection paired with the same test stimuli led to an average decrease of $-57.6 \pm 6.8\%$ in the stimulated pathway, but a change of only $-13.4 \pm 8.4\%$ in the unstimulated pathway. The resting membrane potential did not change following stimulation. In a previous study, hyperpolarizing current applied during high-frequency synaptic stimulation blocked LTP, but LTD of the synaptic input was not reported¹⁶. The input stimulus, however, was typically 30 Hz or higher compared to the 5 Hz used in our experiment, so that the dendritic membrane potential during synaptic stimulation was probably significantly more positive at the 30-Hz rate.

of the test input synapses (Fig. 2*b*). There was a marked reduction in the population spike with smaller decreases in the population e.p.s.p. Note that the stimulus patterns applied to each input were identical in these two experiments, and only the relative phase of the test and conditioning stimuli was altered. With these stimulus patterns, synaptic strength could be repeatedly enhanced and depressed in a single slice (Fig. 2*c*). The covariance model⁷ predicts that stimuli with no covariance should produce no change in synaptic strengths. A test input containing an equal number of in phase and out of phase stimuli mixed together produced no net change in synaptic strength in five slices. Thus, the associative LTP and LTD mechanisms seem to be balanced in both strength and duration.

The simultaneous depolarization of the postsynaptic membrane and activation of glutamate receptors of the N-methyl-D-aspartate (NMDA) subtype leads to LTP induction¹¹⁻¹³

Associative LTP is thought to depend on the spread of current from conditioning synapses to test synapses in the dendritic tree²⁻⁴. Consistent with this hypothesis, we found that the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (AP5, 10 μ M) blocked the induction of associative LTP by the in phase stimuli in CA1 pyramidal neurones (the average change in population spike amplitude, only $-7.3 \pm 10.4\%$ in five slices). By contrast, the application of AP5 to the bathing solution at the same concentration did not affect associative LTD (average change in population spike amplitude, $-42.9 \pm 5.7\%$ in five slices). Thus, the induction of associative LTD does not require the activation of the NMDA receptor.

In a second series of experiments, intracellular recordings from CA1 pyramidal neurones were made using standard techniques¹⁴. An in-phase stimulus produced an increase in peak e.p.s.p. amplitude ($49.2 \pm 7.1\%$ in six cells) and a lowered action potential threshold in the test pathway, as reported previously⁴. Conversely, an out-of-phase stimulus elicited a long-lasting reduction of the e.p.s.p. ($-29.4 \pm 5.5\%$ in six cells) and reduced ability of the test input to elicit firing of action potentials. As in the extracellular experiments, the test input alone produced no long-lasting alterations in intracellular e.p.s.ps or firing properties, whereas the conditioning input alone yielded specific increases of the e.p.s.p. of the strong pathway without altering e.p.s.ps elicited by test input stimulation.

A test stimulus that is out of phase with a conditioning stimulus, arrives when the postsynaptic neurone is hyperpolarized as a consequence of inhibitory postsynaptic potentials and after hyperpolarization from mechanisms intrinsic to pyramidal neurones. This suggests that postsynaptic hyperpolarization coupled with presynaptic activation may trigger LTD. To test this hypothesis, we injected current through intracellular microelectrodes to hyperpolarize or depolarize a CA1 pyramidal neuron while stimulating a synaptic input. Pairing the injection of depolarizing current with the test input led to LTP of those synapses (Fig. 3*a*), whereas a control input inactive during the stimulation did not change, in agreement with previous experiments in which a higher stimulus frequency was used¹⁵⁻¹⁷. Conversely, prolonged, hyperpolarizing current injection, paired with the same test stimuli, led to induction of LTD in the stimulated pathway (Fig. 3*b*), but not in the unstimulated pathway. The application of either depolarizing current, hyperpolarizing current, or the test 5 Hz synaptic stimulation alone did not induce long-term alterations in synaptic strengths. Thus, hyperpolarization and simultaneous presynaptic activity is sufficient for the induction of LTD in CA1 pyramidal neurones. Further experiments are underway to determine whether this mechanism can fully account for associative LTD observed with out of phase inputs.

These experiments identify a novel form of hebbian synaptic plasticity in the hippocampus and confirm predictions made from a covariance model of information storage in neural networks^{7,8}. Unlike previous reports of synaptic depression in the hippocampus^{2,3,9}, the plasticity is associative, long-lasting, and is produced when presynaptic activity occurs while the postsynaptic membrane is hyperpolarized. The chronic hyperpolarization of neurones in visual cortex leads to a long-term depression of the active, but not inactive, inputs from the lateral geniculate nucleus¹⁸. Thus, the hebbian covariance mechanisms present in the hippocampus may also be found elsewhere in the central nervous system^{19,20}. □

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Local positional cues in the neuroepithelium guide retinal axons in embryonic *Xenopus* brain

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GROWING retinal axons home to their distant target, the tectum, even when they are displaced from their normal pathway¹. This argues for long-range guidance mechanisms in the embryonic brain. Growth cones may orientate to diffusible attractants released from the target, as proposed in other systems^{2,3}, or they may use a stable distribution of positional information in the neuroepithelium¹. To distinguish between these possibilities, small pieces of the presumptive optic tract, through which retinal axons will normally grow, were rotated by ~90° either clockwise or counterclockwise. When the retinal axons later encountered the rotated neuroepithelium, they also turned clockwise or counterclockwise, in correspondence with the direction of rotation. This demonstrates that long-range navigation of retinal axons in the vertebrate brain is based partly on stable, local positional factors, rather than on remote diffusible factors.

The general protocol for these experiments is outlined in Fig. 1. After exposing the embryonic brain by removing the epidermis and the optic vesicle on the right side, a piece of neuroepithelium, about 100 µm in diameter, containing ~200 cells of the right presumptive optic tract from a stage 26-28 *Xenopus* embryo⁴ (before axons leave the eye⁵) was excised, labelled with Hoechst dye (Hoechst no. 33258, Sigma), and reimplanted either in the normal orientation (controls), or in rotated orientations (experimentals). The right optic vesicle and epidermis were re-attached and the animals were allowed to recover until they reached stage 37/38-41 (when axons first reach the target⁵), at which time they were fixed, and the retinal fibres originating from the left eye were labelled with DiI (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes), a fluorescent lipophilic dye. DiI has recently been shown to be an excellent marker for labelling axonal projections in the post-mortem brain⁶. To test for the stability of graft orientation, a small crystal of DiI was used to mark a corner of the transplant in a series of experiments (Fig. 2a). It was found that 37 of 44 grafts clearly maintained their grafted orientations (Fig. 2b). Of the remaining seven, the orientations of four were ambiguous, and the other three seemed to have healed into the host in an unintended orientation. Whether the transplant healed incorrectly, or whether the crystal of DiI was dislocated in these cases is not certain. What is clear, however, is that in most cases (≥84%) the graft seemed to maintain its operated orientation. For the experimental series, DiO (3,3'-dioctadecyloxycarbocyanine perchlorate; Molecular Probes) or

carbon particles were used as the orientation markers, in order not to interfere with the fluorescence of the DiI-labelled fibres. DiO crystals and carbon particles, even though they were reliable during the initial transplantations, were not useful as long-term orientation markers. The carbon particles become indistinguishable in the background of developing pigment cells; the DiO crystals were usually extruded from the neuroepithelium, as DiO apparently does not stick to the tissue as well as DiI. Whole-mounted embryonic brains with early optic tract rotations revealed normal surface contours. Rarely (<10% of cases), the rotations produced minor morphological aberrations in the developing brain, probably as a result of poor wound healing; these were discarded from the analysis.

Axons growing through control transplants (that is, removed, stained and reimplanted in their normal orientation) usually grew towards the tectum along trajectories that seemed similar to the trajectories taken by axons in unoperated animals (compare Figs 2c and d and Fig. 3a), although no quantitative comparison between these two conditions was made. Axons growing through rotated transplants (Figs 2e, f and 3c, d, e), however, veered clockwise or counterclockwise on entering the rotated tissue. Quantitative analysis of the results (Fig. 4) showed mostly non-deflected trajectories in operated control animals, whereas experimental groups showed trajectories that deviated in the predicted directions. Often the pathway deflections were

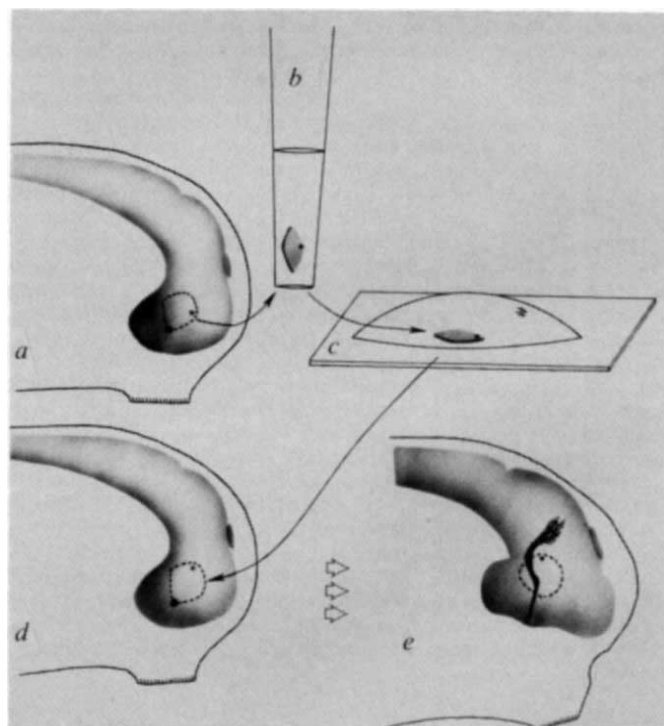
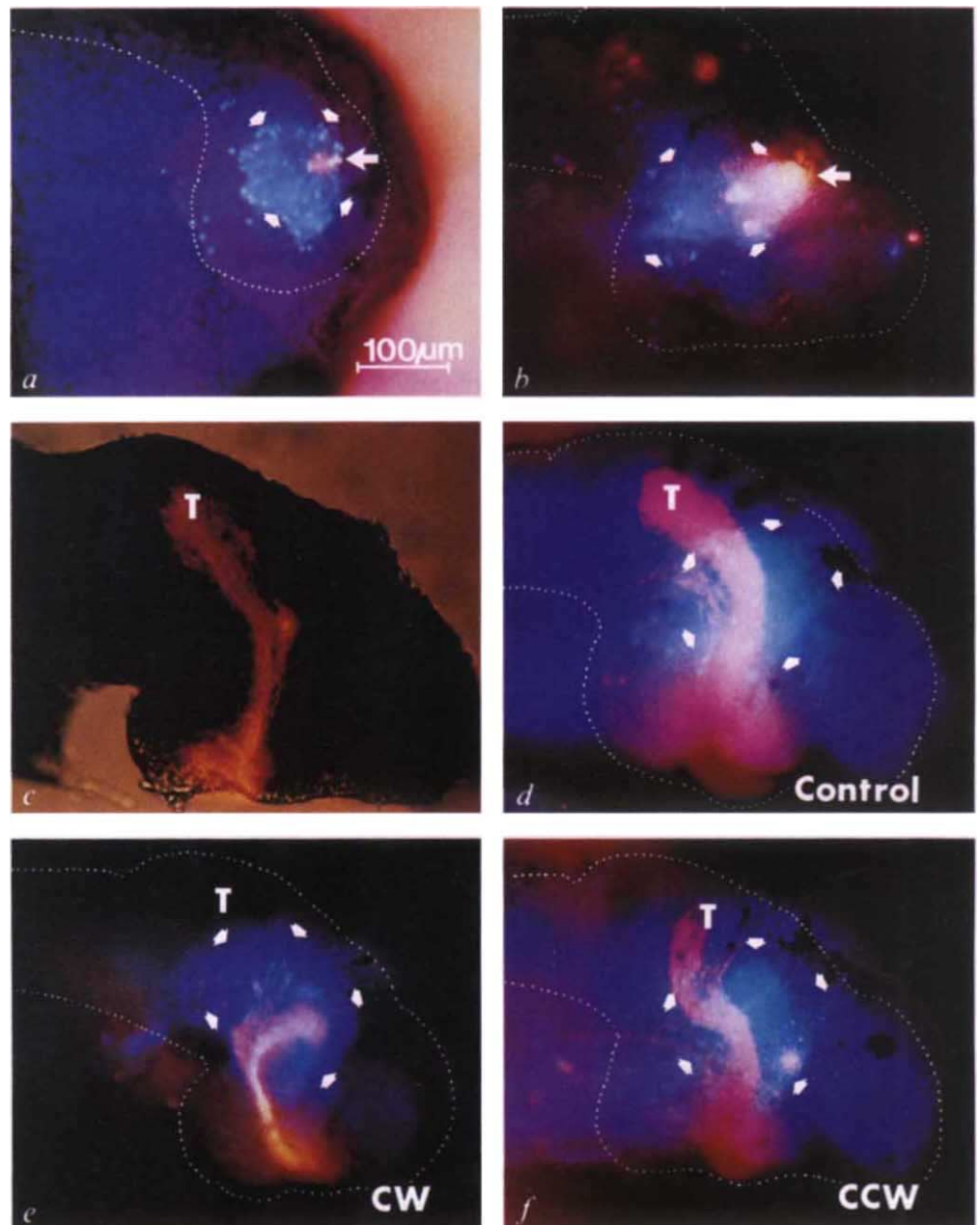


FIG. 1 Procedure of rotation experiment. a, Right side of stage-26 embryonic brain (shaded object) at time of surgery. Dashed lines indicate region of graft to be removed. Star indicates orientation marker; b, Spemann pipette transferring tissue to labelling solution; c, graft incubating in drop of Hoechst dye; d, graft in new counterclockwise orientation; e, optic fibre trajectory through graft.

METHODS. To mark the orientation of the graft, a small crystal of DiO, DiI or a carbon particle was embedded into the anterior corner of the diamond. Staining was in 200 µM Hoechst in Steinberg's solution¹⁷ for 10 min. The tissue was washed twice and repositioned in the brain either in its normal position (controls) or rotated approximately 90° clockwise or counterclockwise (experimentals). After a survival period of up to two days, the embryos were fixed in 4% paraformaldehyde and the optic fibres growing out of the left eye were labelled by placing crystals of DiI on the fixed retinal surface⁶. All samples were photographed as single and double exposures to show the relationship of the Hoechst (graft) and DiI (fibres) fluorescence, and then traced from projected slides to reconstruct line drawings for further analysis.

FIG. 2 Fluorescence micrographs of control and experimental animals. *a*, Control transplant immediately after the operation. Grafted tissue with fluorescent blue cells (indicated by small arrowheads) were observed and photographed. The red spot in the anterior corner of the transplant, indicated by the large arrow, is a crystal of Dil. Dotted lines in this and other panels outline the position of the brain. *b*, Same transplant as in *a*, ~2 days later. It has healed into the brain, retained its Hoechst blue fluorescence, and clearly maintained its orientation, although the Dil fluorescence has intensified and spread; *c*, Dil-labelled retinal fibres in the optic pathway of a stage-40 unoperated animal. The brain of this animal was not labelled and so it was not photographed with the Hoechst fluorescence. (T, approximate location of the tectum.) *d*, Retinal fibres in a control experimental animal. Notice that the fibres have a projection that is similar to that in unoperated animals, even when they enter the blue transplanted tissue (perimeter marked by white arrows); *e*, Fibres travelling through a clockwise (CW) rotated transplant turn clockwise as they enter the graft. *f*, Fibres travelling through a counterclockwise (CCW) rotated transplant turn counterclockwise as they enter the graft, and then reorientate towards the tectum on leaving the graft. Red fluorescent spot near the arrow at the five o'clock edge of the graft (in *f*) is an artefact arising from a stray crystal of Dil, not an orientation marker.



dramatic, approaching 90° (Fig. 3c). The data presented in Fig. 4 are based on the angular deflection (compared with the expected trajectory) achieved by the fibres half-way through the graft. This parameter can be measured reproducibly and objectively, although it is obviously not an indicator of the maximum deflection. The measure clearly shows that all 16 clockwise rotations lead to clockwise deflections of fibres, whereas 12 of 14 counterclockwise rotations lead to counterclockwise deflections (Fig. 4). The curved, rather than kinked, trajectories seen in many of the experimental animals indicate that these growing axons may not easily be capable of making abrupt changes in direction. That the 'momentum' of growing axons in a rotated field leads them to graceful turns rather than to kinks has been suggested previously⁷. Another possibility is that axons are guided, to some extent, by diffusible chemotropic cues emanating from the tectum. If the chemotropic cues are less potent than the stable local cues, this could also explain why the axons are deflected, but do not make sharp 90° kinks when they enter the clockwise and counterclockwise grafts. There were a few

cases in which the optic fibres did not grow through the transplanted region. This was usually because the transplant was too anterior (Fig. 3b). When fibres grew close to, but not into, rotated tissue, they were not deflected.

It is possible that what is being rotated in this study is partly a mechanical pathway, for instance a set of open channels. But, if this were the only factor, 180° rotations should produce similar effects to unrotated control transplants. Rotations of 180° , however, often (11 out of 14 cases) block or severely disorganize fibre outgrowth through the transplants (Fig. 3f). In a few cases (3 out of 14) fibres seemed undeflected as they grew through such transplants. In 2 of the 30 experimental cases, optic fibres entering a transplant that was rotated counterclockwise made a turn in the opposite clockwise direction. These observations are consistent with a previous finding that axons can grow in both directions along the optic tract⁸. Thus, in addition to stable positional markers that influence the direction of axon growth, there may be physical features of the early optic tract that act to support optic axon outgrowth. As ectopic axons can home

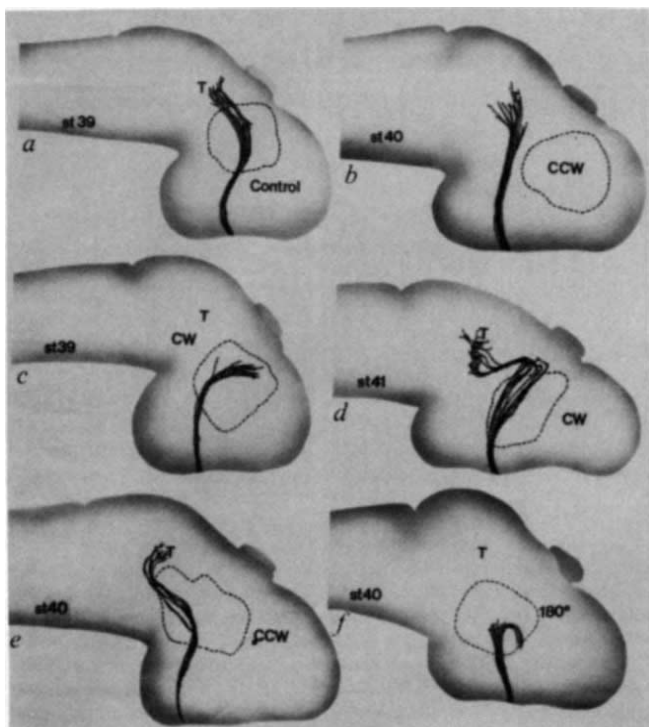
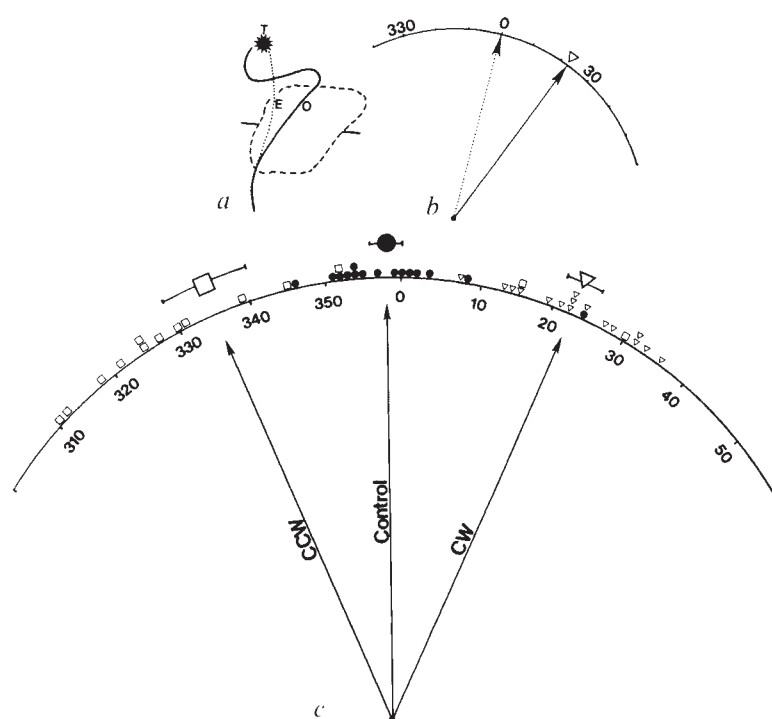


FIG. 3 Reconstructed drawing from fluorescent micrographs of fibres growing through a series of optic tract transplants outlined by dashed lines. *a*, Control experiment (stage 39); *b*, fibres pass close to, but not through, a counter-clockwise transplant and are not deflected (stage 40); *c* and *d*, two examples of fibres growing through clockwise grafts: *c* is a brain from a stage-39 animal and the fibres have not yet left the graft, and *d* is the brain of a stage-41 animal. The telencephalon is more developed here, and the fibres have left the graft and reorientated to the tectum; *e*, example of fibres growing through a counter-clockwise graft (stage 40); *f*, fibres entering, but unable to grow through, a 180°-rotated graft in a stage-40 animal. Some fibres are seen to turn back. T, quantitative estimate of the position of the tectal neuropil based on external features of the brain. Methods are described in detail in ref. 1.

FIG. 4 *a*, Solid line, marked O, represents the average observed trajectory of fibres as they grow through the transplant to their target. This is a re-representation of the case shown in Fig. 3*d*. Dotted line, marked E, estimates the expected path of fibres if they had not entered a transplant; *b*, the tangent to the expected line (E) was assigned a value of 0°, and the tangent to the line derived from the observed fibres (O) is shown relative to this; *c*, results from all cases are presented. The angle of the observed tangents is very close to 0° in control operations (circles) (average $358^\circ \pm 2^\circ$). Clockwise rotations (triangles), however, resulted in deflections generally of about 10–40° (average $22^\circ \pm 2^\circ$ s.e.m.), while counter-clockwise rotations (squares) usually resulted in deflections from 310–340° (average $336^\circ \pm 6^\circ$ s.e.m.). There were 2 of 14 CCW cases with CW deflections.

METHODS. The average observed trajectory (O) of fibres was obtained by plotting a line along the middle of the fibre bundle. The estimated trajectory (E) was constructed with a flexible ruler that exactly matched the observed line of entry into the transplant, but from there was constructed simply to curve gently and directly to the target. The position of the estimated tectal neuropil¹ is marked with a star, labelled T. The deflection produced by the transplant was assessed by measuring the angle between tangents to these two trajectories taken at the dorso-ventral midpoint of the experimental fibres' traverse through the graft (horizontal line at the edges of the graft outline in *a*). By the Watson-William's¹⁸ test for differences in mean angles and by the Mardia-Watson-Wheeler¹⁸ test for differences in angle samples, all three groups (CCW, operated controls, and CW) were significantly different from each other ($P < 0.005$ in all cases).



in on their targets outside the optic tract¹, however, these features of the optic tract are clearly not needed for target-directed axonal navigation.

Retinal axons first enter the contralateral ventral diencephalon (the site of ventral edge of the transplant) at stage 33/34, six to eight hours after the embryonic surgery⁵. Normally, fibres reach the dorsal diencephalon (the site of the dorsal edge of the transplant) at stage 37/38⁵. Many experimental animals were examined at stages 40–41, after optic fibres had grown out of the transplanted zone. In these cases, on leaving the rotated piece of tissue, the retinal fibres readjusted their course and headed towards the tectum (Figs 2*f* and 3*d,e*).

Factors released or extracted from tecta have been shown to influence the survival of retinal ganglion cells in culture^{9–11}. Depriving ganglion cells of their targets *in vivo* also leads to increased cell death¹². Both of these results argue for tectal trophic factors. In contrast, attempts to show that the tectum releases a diffusible orientating factor have been unsuccessful¹³. The present data do not exclude the possibility that the local cues to which the axons respond are molecules that were secreted by the tectum at stages before stage 26, and subsequently stabilized by binding to extracellular matrix components. However, in *Xenopus* and other amphibian embryos in which the tectum is removed at stage 24, retinal axons follow the optic tract to a 'phantom target' before turning in an inappropriate direction^{14,15}. This indicates that guidance cues in the optic pathway exist long after the tectum is removed. Similarly, optic axons in amphibians and chicks do not adjust to a shifted position of the target until they are near enough to interact with it directly^{15,16}. These results indicate that the guidance cues for retinal fibres in the optic tract of the vertebrate brain are target-independent and that the target may exert only a short-range attraction. The experiments reported here strongly support this idea and further show that growing retinal fibres can react to local positional information in the embryonic brain. The same distribution of positional information on the neuroepithelium could be interpreted differently by growth cones from other origins and be used to guide their axons to various distant targets in the brain. □

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High-affinity binding of staphylococcal enterotoxins A and B to HLA-DR

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STAPHYLOCOCCAL enterotoxins A-E (refs 1-3), toxic shock toxin (TST-1) (ref. 1), a product of *Mycoplasma arthritidis*⁴ and the Mls antigens⁵⁻⁷ provoke dramatic T-cell responses. All are extremely potent polyclonal mitogens stimulating a large proportion of both murine and human CD4⁺ and CD8⁺ T cells although activity is tightly restricted by major histocompatibility complex (MHC) class II antigens^{1,2}. The murine T-cell response to staphylococcal enterotoxin B (SEB) has recently been shown to involve only those T cells expressing T-cell receptor V β 3, 8.1, 8.2 and 8.3 domains⁷, a situation which closely mimics the response to Mls antigens^{5,6,8}. This paper examines the initial events in SEA and SEB T-cell activation and shows that MHC restriction results from a direct high affinity binding by intact SEA and SEB to the same site on MHC class II HLA-DR antigens.

Human B- and T-cell lines expressing either class I or class II MHC or both, were examined for their ability to bind radiolabelled SEA and subsequently activate unprimed resting human peripheral T cells (Table 1). Binding and presentation of SEA was clearly dependent on the expression of class II antigens. SEA did not bind to Jurkat and HPB-ALL which both express T-cell receptor molecules (TCRs). Binding of SEA was strongly dependent on temperature being ten-fold higher at 37 °C and 18 °C than at 4 °C. This suggested critical differences in the nature of either the receptor or ligand between these temperatures.

Class II expressing cells were incubated in the presence of [¹²⁵I]SEA at 37 °C, washed, lysed and immunoprecipitated with monoclonal antibodies (mAbs) to MHC antigens (Fig. 1A). High levels of radiolabelled SEA (relative molecular mass M_r 27,000) co-precipitated with HLA-DR using mAb LB3.1 (ref. 9) (lanes a2 and b2) and in quantitative terms this represented 50% and 30% of the total amount bound on an EBV-transformed B-cell line (Normal_{EBV}) and GM4672A cells respectively (lanes a1 and b1). In contrast, little [¹²⁵I]SEA bound to detergent solubilized HLA-DR at any temperature (lane c2) indicating first that SEA did not bind non-specifically to the mAb and second that cell surface associated HLA-DR was conformationally different to detergent solubilized HLA-DR, this difference having a marked effect on its affinity towards SEA. No SEA has yet been detected in immunoprecipitations of

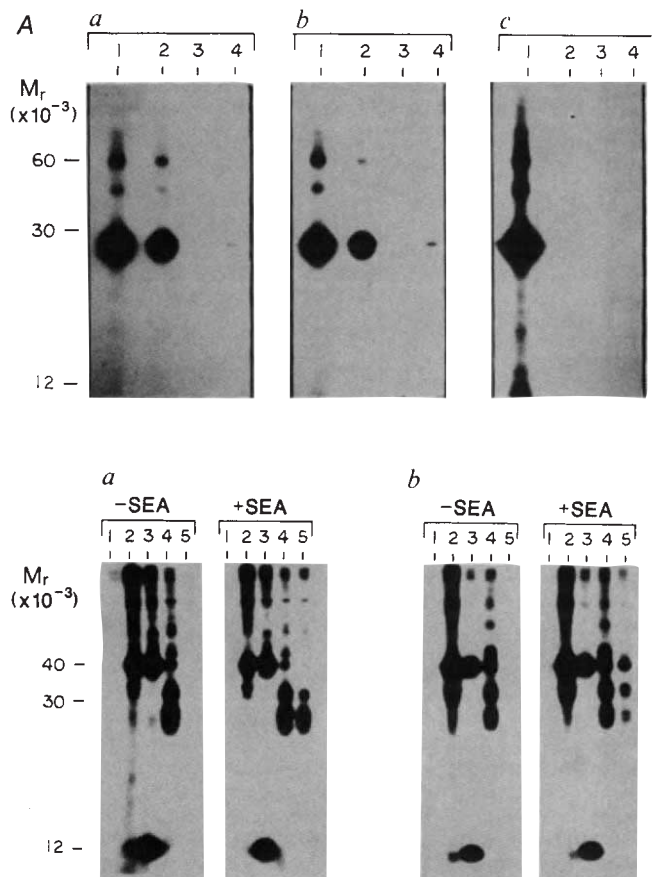


FIG. 1 The primary receptor for SEA is HLA-DR. *a*, [¹²⁵I] SEA, 1 ng; 0.1 μ Ci, labelled by the chloramine T method to 114 μ Ci μ g⁻¹, was incubated with 10⁶ Normal_{EBV} or GM4672A cells for 40 min at 37 °C in RPMI 1640/10% FCS, washed twice with culture media and lysed with 1 ml ice-cold lysis buffer containing 0.5% NP40, 0.3 M NaCl, 10 mM Tris-Cl pH 8.0, 10 mM iodoacetic acid and 1 mM phenyl methyl sulphonyl fluoride (PMSF). Aliquots were immunoprecipitated immediately with: lane 1, rabbit anti-SEA (Serva); lane 2, anti-HLA-DR mAb LB3.1 (ref. 9); lane 3, anti HLA A, B, C monomorphic mAb W6/32 (ref. 10); lane 4, rabbit anti-class I heavy chain (anti-Hc) serum¹¹. *A*, Panel *a*, normal_{EBV} cells; Panel *b*, GM4672A cells; Panel *c*, GM4672A cells lysed with detergent before adding labelled SEA and incubated at 18 °C (shown), 4 °C or 37 °C (not shown but identical results to 18 °C). Precipitates were immobilized on 30 μ L protein A sepharose and washed three times with ice cold lysis buffer before being boiled in SDS sample buffer and run on a 12.5% SDS PAGE gel. *B*, *a*, Normal_{EBV} cells (1 $\times$ 10⁷) and *b*, GM4672A cells (1 $\times$ 10⁷) surface-iodinated using lactoperoxidase as previously described¹⁹ and incubated with or without 10 ng ml⁻¹ unlabelled purified SEA for 40 min at 37 °C. Washed cells were lysed in 1 ml ice cold lysis buffer, precleared twice for 1 h at 4 °C with normal rabbit serum plus fixed *Staphylococcus aureus* bacteria (Pansorbin, Calbiochem). Aliquots were immunoprecipitated with: lane 1, normal mouse serum; lane 2, rabbit anti-Hc serum; lane 3, anti-class I MHC monomorphic mAb W6/32; lane 4, anti HLA-DR mAb LB3.1; lane 5, rabbit anti-SEA serum (Serva). Immobilized precipitates were treated as above and run on a 12.5% SDS PAGE gel.

HLA-DQ or DP (not shown). SEA did not interact with native class I HLA using mAb W6/32 (ref. 10) (lanes a3 and b3) but small amounts readily co-precipitated with class I heavy chain using a rabbit serum specific for denatured class I (devoid of β_2 -microglobulin (anti Hc, ref. 11); lanes a4 and b4). SEA did not bind to class I heavy chain on any of the class II negative cell lines (Table 1). Moreover it could not be detected in anti-Hc precipitations of the class I⁺, class II⁺ Daudi line labelled with SEA (not shown) indicating that this anti-Hc serum does not cross react with class II antigens. These data suggest that SEA, once bound to class II, cross-links a small proportion of class

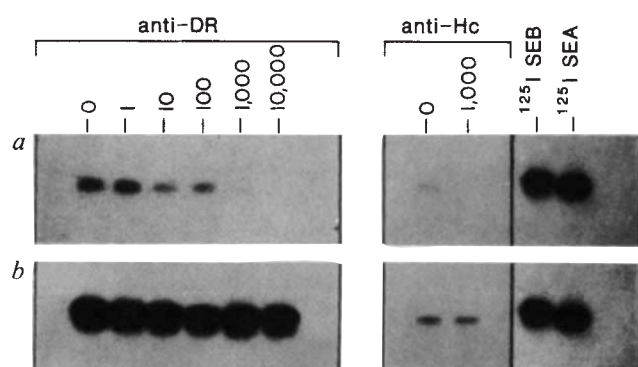


FIG. 2 The binding site on HLA-DR for SEA and SEB is identical. Panel A, Aliquots of 10^5 Normal_{EBV} cells were incubated in 0.5 ml RPMI 1640/10% FCS for 40 min at 37 °C containing 10 ng (2.0 μ Ci) [125 I]SEB (243 μ Ci μ g $^{-1}$) and 0–10,000 ng of unlabelled SEA. Washed cells were lysed and immunoprecipitated with either mAb LB3.1 (HLA-DR) (anti-DR) or rabbit anti MHC class I heavy chain serum (anti-H). Panel B, procedure identical to a except cells were incubated in the presence of 1 ng (0.1 μ Ci) [125 I]SEA (114 μ Ci μ g $^{-1}$) and 0–10,000 ng of unlabelled SEB (Toxin Technology, highly purified).

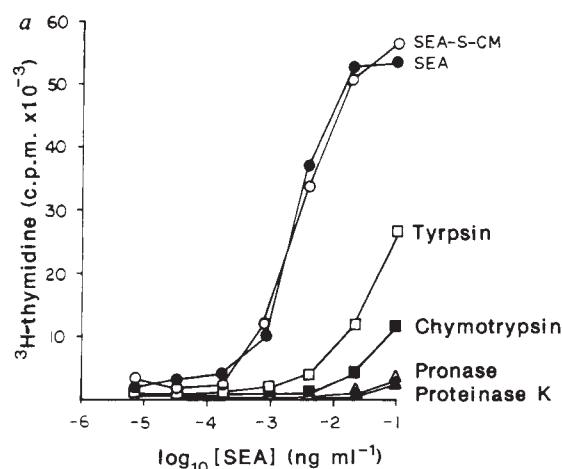
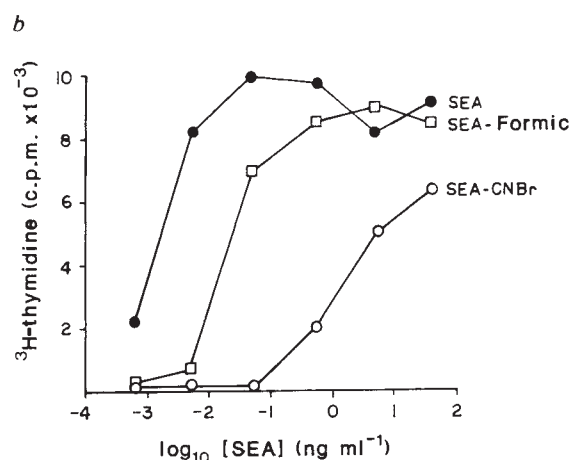


FIG. 3 Proliferative response to fragmented SEA. *a*, SEA (10 μ g) was treated with 10 mM 2-mercaptoethanol in water for 10 min at 20 °C then alkylated in the dark at 4 °C with 12 mM iodoacetic acid pH 7.0. The carboxymethylated SEA (SEA-S-CM) was dialysed and lyophilised. Aliquots (1 μ g) were treated for 2 h at 20 °C in 6 M guanidinium hydrochloride then diluted to 1 M guanidinium HCl. Proteases were added at a ratio of 1:50 with SEA-S-CM and incubated overnight at 37 °C. Treated samples were diluted with 10 ml of culture media, sterile filtered and added to 10^5 peripheral blood lymphocytes at five-fold serial dilutions. Proliferation was determined after 3 days with the addition of 0.5 μ Ci [3 H]thymidine (90 Ci mmol $^{-1}$, Amersham).



b, SEA (1 μ g), treated for 24 h at room temperature with 70% formic acid in the presence or absence of cyanogen bromide (Sigma) was compared over serial five-fold dilution with native SEA for the ability to stimulate resting peripheral T cells. Proliferation after 3 days was determined as described above.

TABLE 1 Binding and presentation of SEA by human B lymphoblastoid lines

APC	Type	HLA expression*			[125 I]SEA binding (c.p.m.)†		T-cell proliferation‡, [3 H]-TdR incorporation (c.p.m.)	
		Negative control	Anti-class I	Anti class II	4 °C	37 °C	–SEA	+SEA
JURKAT	T	32	149	33	4,529	5,002	150	189
HPB-ALL	T	35	161	34	3,407	3,239	152	178
U937	M	37	157	34	2,157	3,014	135	1,439
R.F.	B	33	164	38	5,239	4,419	127	1,619
Normal (EBV)	B	43	190	159	10,300	49,443	141	13,693
GM4672A	B	30	135	150	26,730	110,625	215	16,631
Daudi	B	82	82	180	5,789	20,331	345	12,385

* HLA expression was determined by immunofluorescence using mAbs W6/32 (HLA A, B, C monomorphic (ref. 10)) and LB3.1 (HLA-DR monomorphic (ref. 9)). An irrelevant mAb was used to determine autofluorescence. Results are presented as the Channel number of mean fluorescence intensity.

† Cells were formalin-fixed in 1% paraformaldehyde/phosphate buffered saline for 15 min at 20 °C prior to the addition of 1 ng (0.1 μ Ci) [125 I] SEA (114 μ Ci μ g $^{-1}$) then incubated at either 4 °C or 37 °C for 1 h in RPMI 1640 containing 10% fetal calf serum (FCS) after which time they were washed three times and counted directly in a gamma counter. Results are presented as c.p.m. bound per 10^6 cells. There was no difference in binding between fixed and unfixed cells.

‡ Formalin-fixed cells were incubated in the presence or absence of 1 ng ml $^{-1}$ SEA for 1 h at 37 °C in culture media, washed three times and added at 10^3 per well to 10^5 unprimed human peripheral blood lymphocytes (effector: target ratio of 100:1). Proliferative T-cell responses were measured after 3 days as described in Fig. 3. Standard error was approximately 10%.

I heavy chain. Class I association is not essential for activation, however, as Daudi cells, which lack any form of class I, still bind and present SEA to T cells (Table 1).

The reciprocal experiment, using cell surface iodinated B cells incubated with unlabelled SEA is shown in Fig. 1B. Rabbit anti-SEA serum precipitated bands of M_r 28,000 (28K) and 32K from both lines pre-incubated with SEA (+SEA, lanes 5). In the absence of SEA, the anti-serum precipitated nothing (-SEA, lanes 5). Although the 28K and 32K bands differed between the two cell lines, indicating polymorphism, they were identical in relative molecular mass and relative intensity to the respective α and β chains of HLA-DR precipitated with mAb LB3.1 (lanes 4). A third band of 42K characterized as class I heavy chain also co-precipitated from GM4672A cells. But this band was detected in anti-class II precipitations with mAb LB3.1 in the absence of SEA (-SEA, lane 4) so its presence was independent of SEA and peculiar to GM4672A cells.

Although SEB is only 28% homologous to SEA^{12,13} it directly competed for the same site on HLA-DR. Cells bound ten to thirty times more SEA than SEB (not shown), an affinity difference which was reflected in the ease with which SEA displaced [¹²⁵I]SEB (Fig. 2, panel a). By contrast, SEB was ineffective at displacing [¹²⁵I]SEA (Fig. 2, panel b).

Reduction and alkylation of the single disulphide loop and denaturation in 6M guanidinium hydrochloride followed by renaturation had no effect on the ability of SEA to stimulate resting T cells (Fig. 3). However, any form of fragmentation by a variety of proteases abolished activity (Fig. 3a). Cyanogen bromide (CNBr) cleavage of SEA into an 11K N-terminal fragment and a 16K C-terminal fragment also destroyed activity (Fig. 3b). The remaining 0.1% activity represented uncut SEA. These data imply that either the whole SEA molecule or the central region containing the methionine residue cleaved by CNBr is essential for activity. Clearly, however, the mode of action of SEA differs considerably from normal peptide-induced T-cell proliferation^{14,15} and most likely involves a functional domain much larger than a peptide fragment.

Considering SEA and SEB are the same size as one chain of class II, it is unlikely they bind to the peptide groove^{16,17} without masking normal interaction with the TCR (ref. 18). Certainly, SEA alone does not bind to or activate class II⁻ functional T cells in any shape or form (ref. 1, Table 1 and manuscript in preparation) strongly suggesting that SEA does not bind directly to the TCR. It is possible that SEA, SEB and other so-called 'super-antigens' bind a site apart from the peptide groove on class II MHC proteins, structurally modifying that region of MHC class II which normally interacts with a selected V_β region of the TCR, thus mimicking a strong alloreactive response. This interaction would occur irrespective of whether the V_β is expressed on CD4 or CD8 cells.

This paper describes a unique site on class II MHC proteins to which SEA and SEB bind. Preliminary evidence indicates that another bacterial antigen, TST-1, whose sequence bears little similarity to either SEA or SEB, also binds to the identical site. □

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Brefeldin A implicates egress from endoplasmic reticulum in class I restricted antigen presentation

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MOST antigens must be processed intracellularly before they can be presented, in association with major histocompatibility complex (MHC) molecules at the cell surface, for recognition by the antigen-specific receptor of T cells¹⁻³. This processing appears to involve cleavage of protein antigens to smaller peptides⁴⁻⁷. Only certain fragments of any protein can serve as T-cell epitopes and this is, at least in part, determined by the requirement that peptides be able to bind the MHC molecules⁸⁻¹⁰. Class I restricted antigens are derived from proteins, such as viral antigens, that are synthesized within the presenting cell¹¹⁻¹³. Many of these antigens are cytosolic proteins and recent evidence suggests that it is in the cytosol that these proteins are processed to produce either the antigenic peptides or processed intermediates¹³⁻¹⁶. How and where these processed cytosolic antigens cross the membrane of the vacuolar system and bind to the extracellular domain of the class I molecule is not known but one obvious site for this process is the endoplasmic reticulum (ER), because this organelle is specialized to translocate proteins across the membrane from the cytosol into the secretory system. Based on this model, we reasoned that if we could pharmacologically block the movement of proteins out of the ER, endogenous antigen presentation would cease. An agent which causes such an effect is available^{17,18}—the fungal antibiotic Brefeldin A (BFA). Consistent with the above hypothesis, we report that BFA completely abolishes the ability of a cell to present endogenously synthesized antigens to class I restricted cytotoxic T cells.

Previous work has characterized the cellular effects of BFA^{17,18}. Treatment with this drug rapidly and reversibly blocks transport of newly synthesized proteins out of the ER. This change is accompanied by a redistribution of cis/medial Golgi components to the ER. Proteins retained in the ER undergo carbohydrate processing by these recycled cis/medial Golgi enzymes, but do not acquire sialic acid¹⁹, a modification which has been mapped to the trans Golgi system²⁰. In addition, this drug has no apparent effect on endocytosis, endosome acidification, lysosomal function or the trans Golgi system¹⁸. To test the effect of BFA on presentation of antigens by the endogenous pathway, we used cells from the human B lymphoblastoid cell line JY, infected with influenza A/JAP/57 virus, as target cells. Upon viral infection these cells readily present the T-cell epitope corresponding to amino acids 55-73 of the influenza A matrix protein⁷ and they become, as a result, susceptible to lysis by matrix peptide-specific cytotoxic T cells that are HLA-A2 restricted²¹. When target cells are treated with BFA after the initial period of viral infection, however, subsequent

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antigen presentation is greatly inhibited (Fig. 1a, b). Typically, BFA decreases maximal cell-mediated lysis by 96% ($n = 18$).

BFA does not act by blocking cytotoxic T lymphocyte (CTL) function or the ability of JY cells to be lysed by killer cells as it does not inhibit the lysis of infected JY cells pulsed with the synthetic peptide corresponding to amino acids 55-73 of the matrix protein (M1: 55-73) (Fig. 3a). Also, killing of JY cells by alloreactive CTL clones which recognize HLA-A2 is not inhibited (Fig. 3b). Nor does it appear to interfere with the

earliest events in the viral infection as no difference in effect occurs when BFA is added before or 30 or 90 min after the addition of virus (see also Fig. 1a below). When BFA is added at increasingly longer intervals after infection, however, a progressive loss of inhibition of cell-mediated lysis occurs (Fig. 1c). Adding BFA 5.5 h after infection causes [5-35%] inhibition of the maximal killing in different experiments (data not shown), indicating that sufficient antigen is processed beyond the BFA-sensitive step before addition of the drug to activate T-cell

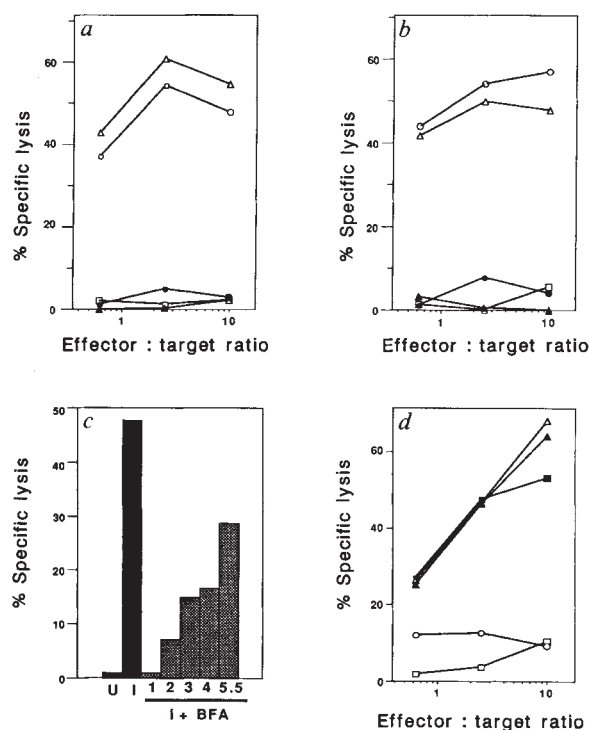


FIG. 1 BFA reversibly blocks class I restricted cell-mediated lysis. a, b, Lysis by the HLA A2 restricted Influenza A specific CTL lines Q115 (a) and Q124 (b). JY target cells: uninfected ($\square$), pulsed with virus for 30 min ($\triangle$), or 90 min ($\circ$), or pulsed with virus for 30 min ($\blacktriangle$), or 90 min ($\bullet$) and then treated with BFA. c, Lysis by cell line Q124 of JY target cells treated with BFA (grey bars) added at increasing intervals (as marked in hours) after addition of influenza A/JAP/57 (I + BFA). Black bars denote uninfected (U) and infected (I) cells not treated with BFA. d, Lysis by cell line Q115 of JY cells either untreated ($\blacksquare$), treated with BFA ($\circ$), or treated with BFA for only 3 h ($\triangle$), or 5.5 h ($\blacktriangle$) after infection. (Uninfected cells $\square$). In all experiments CTL were added 5.5 h after viral infection. BFA treatment was maintained throughout the CTL assay except in (d).

METHODS. CTL lines specific for the influenza A matrix-derived peptide M1: 55-73 (ref. 7) were generated from peripheral blood lymphocytes of the HLA-A2 positive donors Q115 and Q124 as described previously²¹. CTL activity was measured in a standard 4 h ^{51}Cr -release assay³¹. Epstein-Barr virus-transformed human B lymphoblastoid cells (JY) were incubated overnight with ^{51}Cr , washed, resuspended at $4 \times 10^6 \text{ ml}^{-1}$ in RPMI 1640 containing 10% fetal calf serum (RPMI/10), infected with influenza A/JAP/57 (100 μl infectious allantoic fluid per 2×10^6 cells) for 1 h (unless otherwise noted) washed and resuspended at $5 \times 10^5 \text{ ml}^{-1}$ in RPMI/10 $\pm 0.5 \mu\text{g ml}^{-1}$ BFA at 37°C and incubated for a total of 5.5 h after infection. If BFA was added or removed during this period, cells were washed and resuspended in RPMI/10 $\pm 0.5 \mu\text{g ml}^{-1}$ BFA. Washed cells were plated at 5×10^3 per well in 100 μl RPMI/10 $\pm 1.0 \mu\text{g ml}^{-1}$ BFA. CTL cells at the appropriate effector:target ratios were added in 100 μl RPMI/10. After 4 h the supernatants were assayed for ^{51}Cr release and per cent specific lysis was determined from the expression (release by CTL - spontaneous release)/(release by 1% Triton X-100 - spontaneous release) $\times 100$.

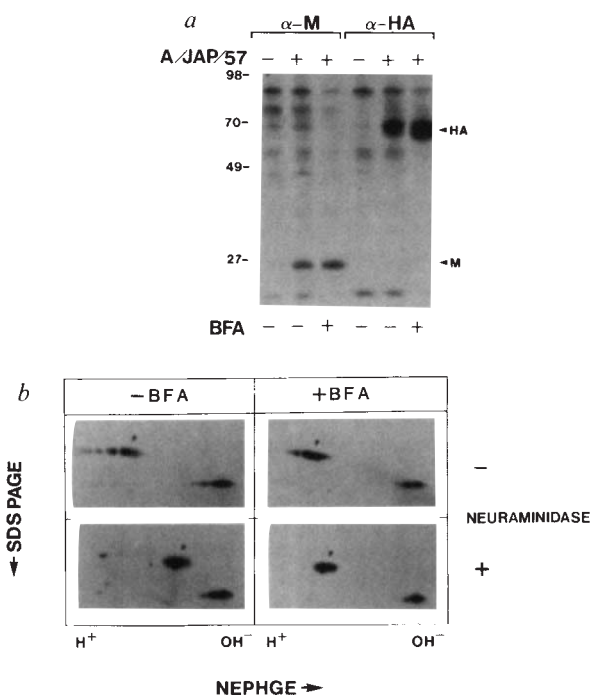


FIG. 2 Biosynthesis and processing of influenza viral proteins in BFA treated cells. a, Immunoprecipitation of matrix (M) and haemagglutinin (HA) from BFA treated (+) and untreated (-) infected JY cells. b, Two-dimensional nonequilibrium pH gradient electrophoresis (NEPHGE)/SDS PAGE analysis of HA (arrowhead) immunoprecipitated from BFA treated or untreated A/JAP/57 infected JY cells in the presence (+) or absence (-) of neuraminidase. Cleavage by neuraminidase of sialic acid residues from the oligosaccharide groups of mature HA in BFA untreated cells results in a shift in migration to a more basic pH. A comparable shift is not seen in BFA treated cells indicating that these chains were not modified by the addition of sialic acid. METHODS. a, JY cells (10^7) were incubated with influenza A/JAP/57 for 1 h, resuspended in RPMI/10 $\pm 0.5 \mu\text{g ml}^{-1}$ BFA at 37°C for 5 h, preincubated for 5 min in methionine-free DMEM containing 10% fetal calf serum, labelled at 10^8 ml^{-1} with 0.6 mCi per ml [^{35}S] methionine (Trans- ^{35}S -label, ICN) for 30 min at 37°C , and solubilized at 4°C in lysis buffer containing a mixture of protease inhibitors as previously described³². Cells were then incubated for 1-2 h with the anti-matrix monoclonal antibody 174/4 (α -M) or six pooled monoclonal antibodies to the H2N2 type HA molecule (α -HA) bound to Goat-anti-mouse-IgG covalently coupled to agarose beads (Cappel) pre-cleared with lysate from unlabelled, uninfected JY cells at 10^8 ml^{-1} . Non-reduced immunoprecipitates were electrophoresed on a 10% SDS-polyacrylamide gel. Relative molecular masses, M_r ($\times 10^{-3}$) are shown at the left side of the figure. b, Cells were infected and labelled as above, then resuspended (2.4×10^7 per 10 ml) in complete RPMI/10, chased for 2 h at 37°C , detergent solubilized, immunoprecipitated with anti-HA monoclonal as described above, heated at 95°C for 5 min in 0.1 M sodium phosphate buffer (pH 6.1) containing 50 mM EDTA and 1% NP-40 and then incubated for 1 h at 37°C in the absence or presence of 10 mU *Arthrobacter urefaciens* neuraminidase (Calbiochem-Behring). Samples were analysed by two-dimensional NEPHGE/SDS-PAGE under reducing conditions as previously described³². The moiety present at higher mobility and more basic pH than HA is a nonspecific precipitate which was observed at equal intensity in immunoprecipitations by irrelevant antibody (data not shown).

killing. The effect of BFA on antigen presentation is completely reversible (Fig. 1d). When the drug is removed from the assay media just before the addition of effector cells, normal levels of cell-mediated lysis occur, implying that the recovery from BFA is rapid compared with the 4 h time course of the CTL assay.

BFA does not significantly inhibit viral protein synthesis (Fig. 2a). Metabolic labelling studies demonstrate that levels of influenza matrix and haemagglutinin (HA) synthesis in BFA-treated cells, 5 h after viral infection, vary between 50 and 120% of those seen in untreated cells. Despite this variability, however, we noted very few instances (2 of 18 experiments) when the level of BFA inhibition of class I-restricted cell-mediated lysis was less than 90%. In addition, BFA did not significantly affect the overall rate of protein synthesis of JY cells nor did pretreatment with BFA interfere with the ability of virus to infect cells (data not shown). These results are complementary to previous work demonstrating that the absolute level of antigen biosynthesis is less important than post-translational processing of the antigen in bringing about efficient antigen presentation^{5,13,17}.

In the normal course of viral infection the HA molecule is co-translationally inserted in the ER membrane. It then undergoes enzymatic processing of its oligosaccharide side chains as it moves through the secretory pathway, eventually acquiring sialic acid in the trans Golgi system^{20,22,23} before being expressed at the cell surface. In the presence of BFA the acquisition of sialic acid on HA is markedly inhibited, as demonstrated by the minimal effect of neuraminidase on the mobility of HA from BFA-treated cells (Fig. 2b). In addition, we find no evidence for transport of newly synthesized HA to the cell surface in the presence of BFA as judged by external proteolysis experiments (data not shown). This is consistent with previous observations on the effect of BFA on carbohydrate processing^{18,19} and provides supporting evidence for the absence of transport of newly synthesized proteins to the trans Golgi system under our experimental conditions.

The inhibition of CTL killing of virally infected cells brought about by BFA appears to be independent of measurable changes in the cell surface concentration of class I MHC molecules²⁴. BFA does not influence class I restricted cell-mediated lysis of cells pulsed with the matrix derived peptide M1:55-73 (Fig. 3a) nor does it affect the lytic function of alloreactive CTLs specific for HLA-A2 (Fig. 3b). Because both of these processes depend upon the population of Class I MHC on the cell surface, these results suggest that BFA does not significantly affect the surface concentration of these membrane bound glycoproteins.

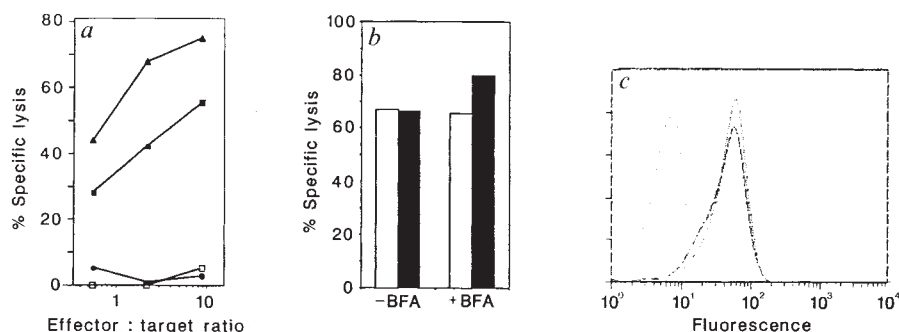


FIG. 3 a, Lysis by CTL line Q115 of A/JAP/57 infected JY cells pulsed with M1:55-73 peptide (—▲—▲—) in the presence of BFA, uninfected (—□—□—), infected (—■—■—), or infected and treated with BFA (—●—●—). b, Lysis of BFA treated (+) and untreated (–) JY cells by the HLA-A2 specific alloreactive CTL clones²¹ IIC5 (white bars) and IA6 (black bars) at effector to target ratios of 10:1. c, Indirect immunofluorescence staining of class I HLA molecules in influenza A infected JY cells, treated with BFA (·····), or left untreated (---). Irrelevant antibody (5C1) used as specificity control (·····).

METHODS. a, JY cells were infected with virus as described in Fig. 1, except that BFA (10 $\mu\text{g ml}^{-1}$) was added 30 min before adding the virus. (We have

In addition, FACS analysis comparing BFA-treated and untreated cells reveals no significant difference in class I surface expression six hours after addition of the drug (Fig. 3c).

Additional experiments were carried out to analyse the effect of BFA on the processing and presentation of exogenous antigens by class II molecules. The soluble protein antigen pigeon cytochrome c must normally undergo proteolytic processing before it can be presented to the antigen-specific class II restricted murine T helper cell hybridoma 2B4 (refs 25, 26). Multiple experiments revealed little or no effect of BFA on the ability of B cells to process and present antigens, and thereby to induce the production of Interleukin-2 (IL-2) by 2B4 T cells. We are currently pursuing the role of intracellular transport processes in class II restricted antigen presentation.

Thus, our results demonstrate that BFA prevents presentation of endogenously synthesized cytosolic antigen to class I-restricted CTL without blocking processes which depend only on surface MHC molecules. Based on our understanding of the effects of BFA on protein export from the ER, these data show that some component of the class I antigen presentation system requires egress from the ER in order to successfully present endogenously synthesized antigen on the cell surface.

These results allow us to propose a working model for the role of the ER in antigen presentation. It is based upon the assumption that the effect of BFA on endogenous, class I restricted antigen presentation is attributable to the inhibition of egress of proteins from the ER. We have shown that BFA does not directly block (1) viral infection, viral or host protein biosynthesis; (2) CTL effector function; (3) the ability of target cells to be killed; or (4) the function of class I molecules in presenting peptides or to act as a stimulus for alloreactive CTL. In other studies we have failed to detect any effect of BFA on ER or lysosomal degradation^{18,19,27} or on the turnover of a number of cytosolic antigens (J. Nuchtern, unpublished results). According to our model, newly synthesized cytosolic antigen, or antigen completely or partially degraded to antigenic peptides or intermediates within the cytosol, is transported across the membrane of the ER, perhaps utilizing the ER translocon which is used to transport newly synthesized proteins²⁸.

Once in the vacuolar system, the class I-binding epitope should function as a secreted peptide, yet it does not appear that antigens are either secreted by the cell or gain access to the endosomal system for binding to class II MHC molecules. For this reason we postulate that once the processed antigen enters the ER it either binds to a class I molecule or is further degraded

not observed any differences in the effects of BFA at this concentration compared to the lower concentrations used in Figs 1 and 2). M1:55-73 peptide was added at the time of viral infection at a final concentration of 10 $\mu\text{g ml}^{-1}$. CTL activity was measured 5.5 h later in a standard 4 h ⁵¹Cr release assay. b, JY cells were treated with or without BFA (10 $\mu\text{g ml}^{-1}$), incubated for 5.5 h at 37 °C, and combined with the allospecific clones in a standard lysis assay. c, The first antibody was the murine monoclonal W6/32 (American Type Cell Culture) (anti-class I HLA, 1:10⁴ dilution of ascites fluid) or 5C1 (nonspecific); the second antibody was FITC conjugated, affinity purified goat-anti-mouse-Ig antibody at 1:50 dilution. The cells were analysed using a modified FACS II (Becton-Dickinson)³³.

by the newly described ER degradation system^{29,30}. We have recently demonstrated that whether or not a protein within the ER is degraded can be directly correlated with the state of assembly of that protein²⁷ and an analogous process for antigen would result in the degradation of any peptide that is not stably bound to class I molecules. The peptide-MHC complex then leaves the ER and travels through the Golgi apparatus to the plasma membrane. This model provides multiple testable hypotheses about the cell biology of endogenous antigen presentation which we are currently examining. □

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Isolation of CD4⁺ CD8⁻ mycobacteria-reactive T lymphocyte clones from rheumatoid arthritis synovial fluid

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THE majority of peripheral T cells express a heterodimeric, α/β T-cell receptor, which recognizes specific antigenic peptides bound to self major histocompatibility complex (MHC) molecules, and either the CD4 or CD8 surface markers. An additional subset of T cells^{1,2}, whose physiological function is unknown, express a distinct CD3-associated receptor composed of γ and δ chains¹. This subset includes cells lacking both CD4 and CD8 surface markers, which may be involved in autoimmunity³⁻⁶. The recognition specificity of the γ/δ receptor is not well characterized and has been defined in only one case to date, a murine cell line which shows MHC-linked specificity⁷. In this report, we describe the isolation of CD4⁺ CD8⁻, γ/δ TCR bearing T cell clones from the synovial fluid of a rheumatoid arthritis patient. These T cell clones respond specifically to mycobacterial antigens without MHC restriction.

Our reasons for cloning these cells in the presence of mycobacterial antigens were twofold. First, synovial cells, although poor responders to mitogens such as phytohaemagglutinin (ref. 8) or anti-CD3 antibodies⁹, have been shown to respond vigorously to mycobacterial antigens^{8,10}. Second, the association between the immune response to mycobacterial antigens and HLA-DR4 (ref. 11), a class II MHC antigen commonly associated with rheumatoid arthritis¹², suggested that this immune response might be related to the pathology of the disease. The proliferative

responses of fresh synovial cells obtained from a 44-year-old patient with early rheumatoid arthritis are shown in Table 1. There was a significant response to the acetone-precipitable fraction of mycobacterium tuberculosis (AP-MT) by synovial fluid cells but not by cells from the peripheral blood.

To enrich for AP-MT reactive T cells, a T-cell line was established from this patient by repeated stimulations of synovial fluid T cells with AP-MT in the presence of irradiated, autologous peripheral blood mononuclear cells. The resultant line, JBT1, had a mixed phenotype. Flow cytometric analysis showed that 73% of the cells were CD4⁺CD8⁻ and 23% were CD4⁻CD8⁻ (Table 1). In unselected synovial fluid the percentage of double negative cells was 11% and in peripheral blood it was 6%. Thus, double negative T cells were found in a higher percentage in the synovial fluid, and proliferated following repeated stimulations with the mycobacterial antigen AP-MT.

We cloned line JBT1 by the limiting dilution technique and propagated fourteen clones successfully. Of the five that have been studied in detail, four were found to be double negative, whereas the fifth was found to have the CD4⁺CD8⁻ phenotype. Figure 1 shows the fluorescence profiles of the CD4⁺ clone 1.1 and a representative double negative clone designated 1.2. All clones expressed CD2 and CD3 surface markers as well as DR antigens and IL-2 receptors but the double negative clones expressed neither CD4 nor CD8 (Fig. 1). Both types of clones did not stain positively with the following monoclonal antibodies: anti-Leu 7 or anti-Leu 9 (NK markers), anti-Leu 8 (suppressor inducer marker), anti-Leu 12 (B cell marker) (data not shown). Whereas the CD4⁺ clone stained positively with anti-Leu 1 (CD5), the double-negative clones showed either dull or negative staining with this monoclonal antibody (not shown).

The surface expression of T-cell receptor (TCR) chains was examined by staining with monoclonal antibodies. These included WT31, specific for an α/β TCR framework determinant¹³; Ti- γ A, specific for TCR γ -chain¹⁴; and two monoclonal antibodies specific for TCR δ -chain, δ TCS1 (ref. 15) and TCR δ 1 (ref. 16). Flow cytometric analysis (Fig. 2a) showed that clone 1.1, a CD4⁺ clone, had an α/β TCR, whereas the four double negative clones expressed γ/δ heterodimers, as indicated by positive staining with both Ti- γ A and TCR δ 1 and no staining with WT31 antibody (Fig. 2a). The δ TCS1 antibody, which binds many γ/δ bearing cells¹⁵, did not stain any of our γ/δ T cell clones (Fig. 2a), although it stained PEER cells (not shown). It is conceivable that our double-negative clones express a different V δ gene product.

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Immunoprecipitation analysis using anti-peptide antisera was carried out to confirm that the CD3-associated TCR complexes expressed by the 1.2, 1.3, and 1.6 T cell clones represent γ/δ TCR complexes (Fig. 2b). Both the anti-TCR- γ and anti-TCR- δ sera precipitated the CD3-associated TCR, indicating that this receptor is a γ/δ TCR. Because both antisera precipitate the γ/δ complex it is not possible (without results of further experiments which are in progress) to determine which of the observed bands is γ or δ .

The increase in the percentage of double-negative T cells following repeated restimulations of the synovial fluid T cells with the mycobacterial antigen AP-MT (Table 1) suggested that the double-negative cells proliferated in response to AP-MT. To test this possibility, we measured proliferation of different clones to AP-MT using the thymidine incorporation assay (Table 2). Both clone 1.1 of the $CD4^+$, α/β TCR phenotype, and the four double-negative γ/δ TCR clones proliferated in response to AP-MT. This proliferation was antigen-specific as the γ/δ

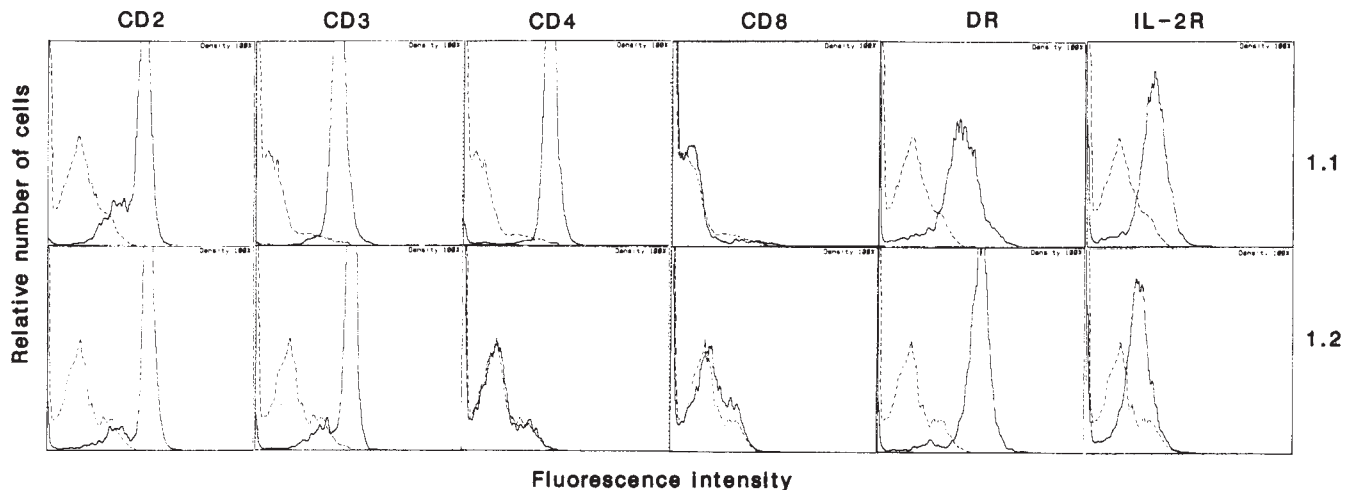


FIG. 1 FCM analysis of the surface marker phenotype of two representative clones. Five out of the fourteen clones obtained were studied more extensively. One clone, 1.1, was found to be of the $CD4^+$ phenotype and four clones, 1.2, 1.3, 1.4 and 1.6, had the double-negative phenotype represented here by clone 1.2. Fluorescence intensity (x axis) as a function of relative cell number (y axis) of test antibodies (solid line) superimposed on background fluorescence intensity obtained with isotype matched control antibodies (broken line) are shown.

METHODS. The JBT1 T cell line was cloned by the limiting dilution technique based on the method described by Ottenhof *et al.*²¹, with slight modifications. Briefly, viable JBT1 cells purified by Ficoll gradient were diluted to give 0.5 cells per 0.1 ml and plated into flat-bottom 96-microtitre plates (Costar), at

0.1 ml per well over a feeder cell mixture, which consisted of autologous irradiated EBV-transformed B cells (10^5 cells ml^{-1}), peripheral blood mononuclear cells from three random donors (10^6 cell ml^{-1}) and AP-MT ($10 \mu g$ ml^{-1}). Twenty-one wells contained growing cultures out of 96 wells originally seeded. Of these, fourteen clones were successfully propagated by repeated weekly cycles of restimulation with the feeder/antigen mixture, followed by expansion in IL-2-containing medium. The phenotypic analysis was done 7–10 days after restimulation. Indirect flow cytometric analysis included the following monoclonal antibodies at the first stage: OKT 11 (anti-CD2, Ortho Diagnostics), anti-Leu 4 (CD3), anti-Leu 3 (CD4), anti-Leu 2 (CD8), L243 (anti-DR), anti IL-2 receptor (all from Becton-Dickinson). For second stage, FITC-conjugated goat anti-mouse IgG (Tago) was used.

TABLE 1 Proliferative responses and surface phenotype of peripheral blood, synovial fluid mononuclear cells, and the JBT1 anti-AP-MT cell line

Cells derived from	Proliferative response (stimulation index $\pm$ s.d.)		Surface phenotype of T cells (% positive cells)				
	AP-MT	PHA	$CD4^+$	$CD8^+$	$CD4^-CD8^-$	TCR α/β	TCR γ/δ
Peripheral blood	3.2 ± 0.1	63.7 ± 0.6	67	17	6	69	4
Synovial fluid	22.3 ± 0.5	25.3 ± 0.6	49	40	11	62	11
JBT1 Line	19.0 ± 0.2	39.5 ± 0.4	73	0	23	70	ND

Mononuclear cells were separated from fresh heparinized synovial fluid and peripheral blood on Ficoll-Hypaque gradients. The JBT1 anti-AP-MT T cell line was established by incubating synovial fluid mononuclear cells (10^6 ml^{-1}) with AP-MT (prepared as previously described¹⁰; $10 \mu g$ ml^{-1}) in RPMI 1640 medium supplemented with 10% pooled normal human serum in 24-well plates (Costar). After 5 days, the blasts were resuspended for 7 days in medium containing $10 U$ ml^{-1} recombinant human IL-2 (Cetus), and then restimulated for 3 days with AP-MT in the presence of irradiated (3,000 R) autologous peripheral blood mononuclear cells in IL-2-free medium. After eight cycles of restimulation followed by expansion in IL-2, proliferation and phenotype were analysed. Proliferative responses of peripheral blood and synovial fluid mononuclear cells were measured as described¹⁰, incubating with AP-MT ($10 \mu g$ ml^{-1}) for 5 days or phytohaemagglutinin (Wellcome Diagnostics $1 \mu g$ ml^{-1}) for 3 days. Proliferative responses of the JBT1 line were measured by incubating 15×10^3 T cells and 75×10^3 irradiated autologous peripheral blood mononuclear cells per well with or without AP-MT ($10 \mu g$ ml^{-1}) or phytohaemagglutinin ($0.25 \mu g$ ml^{-1}) for 3 days. [3H]thymidine ($1 \mu Ci$ per well) was added to all cultures 18 h before cell collection. Results are expressed as stimulation index (ratio of mean c.p.m. with antigen or mitogen/mean c.p.m. without antigen or mitogen). Control values for cultures without antigen or mitogen were 465 ± 31 at 3 days and 514 ± 177 at 5 days for peripheral blood; 911 ± 168 at 3 days and 346 ± 73 at 5 days for synovial fluid mononuclear cells; and 510 ± 64 for cell line JBT1. To determine the phenotype of the cells, synovial fluid mononuclear cells and simultaneously drawn peripheral blood mononuclear cells were enriched for T cells using the E-rosette technique. Viable JBT1 cells were purified over Ficoll gradients. For two-colour analysis, the following directly conjugated antibodies were used: anti-CD4 (Leu 3-FITC), anti-CD8 (Leu 2-FITC), anti-CD3 (Leu 4-PE) (all from Becton-Dickinson) and directly conjugated isotype-matched control antibodies (provided by Dr L. Herzenberg). Cells were stained with each of these antibodies alone and with combinations of anti-CD3 + anti-CD4, anti-CD3 + anti-CD8 and anti-CD3 + anti-CD4 + anti-CD8. For indirect immunofluorescent stainings, cells were stained with the following antibodies: anti- α/β TCR (WT31, Becton-Dickinson), anti-TCR γ -chain (Ti- γ A, a generous gift of Dr T. Hercend¹⁴), or an isotype-matched control antibody, followed by FITC-conjugated goat anti-mouse IgG (Tago). Flow cytometric analysis was performed using a B-D single Argon ion laser (488 nm) FACS III equipped with filters for fluorescein and phycoerythrin, interfaced to a Digital Equipment Corporation microVAX computer, using the FACS DESK software. ND, not determined.

clones did not proliferate in response to myelin basic protein (Table 2) and various other control antigens (not shown). Although AP-MT is a relatively crude antigen, it is not mitogenic as indicated by its failure to stimulate both BF1 (γ/δ) and 8.1 (α/β) control T cell clones derived from the peripheral blood of a normal individual (Table 2). BF1 and 8.1 are representative of γ/δ and α/β alloreactive clones, respectively, which recognize class I MHC antigens¹⁶. We are currently analysing the fine antigenic specificity of the double-negative clones. One clone (1.6), proliferated in response to P64, a biochemically purified heat-shock protein of relative molecular mass 64,000 from mycobacterium bovis BCG (Table 2).

The CD4⁺ clone and the four double-negative clones differ in their MHC restriction pattern. As shown in Fig. 3, the CD4⁺ clone 1.1 proliferated only in the presence of autologous antigen-presenting cells and its proliferation could be blocked by anti-DR antibodies (60% suppression). In contrast, the double negative clones, represented in Fig. 3 by clone 1.3, proliferated in the presence of antigen-presenting cells obtained from different unrelated individuals. This proliferation could not be blocked by anti-class I or class II HLA antibodies. HLA class I-negative Daudi cells could present AP-MT to the double negative clones, whereas K562 cells, which do not express both class I and class II MHC antigens, could not. No proliferative response to AP-

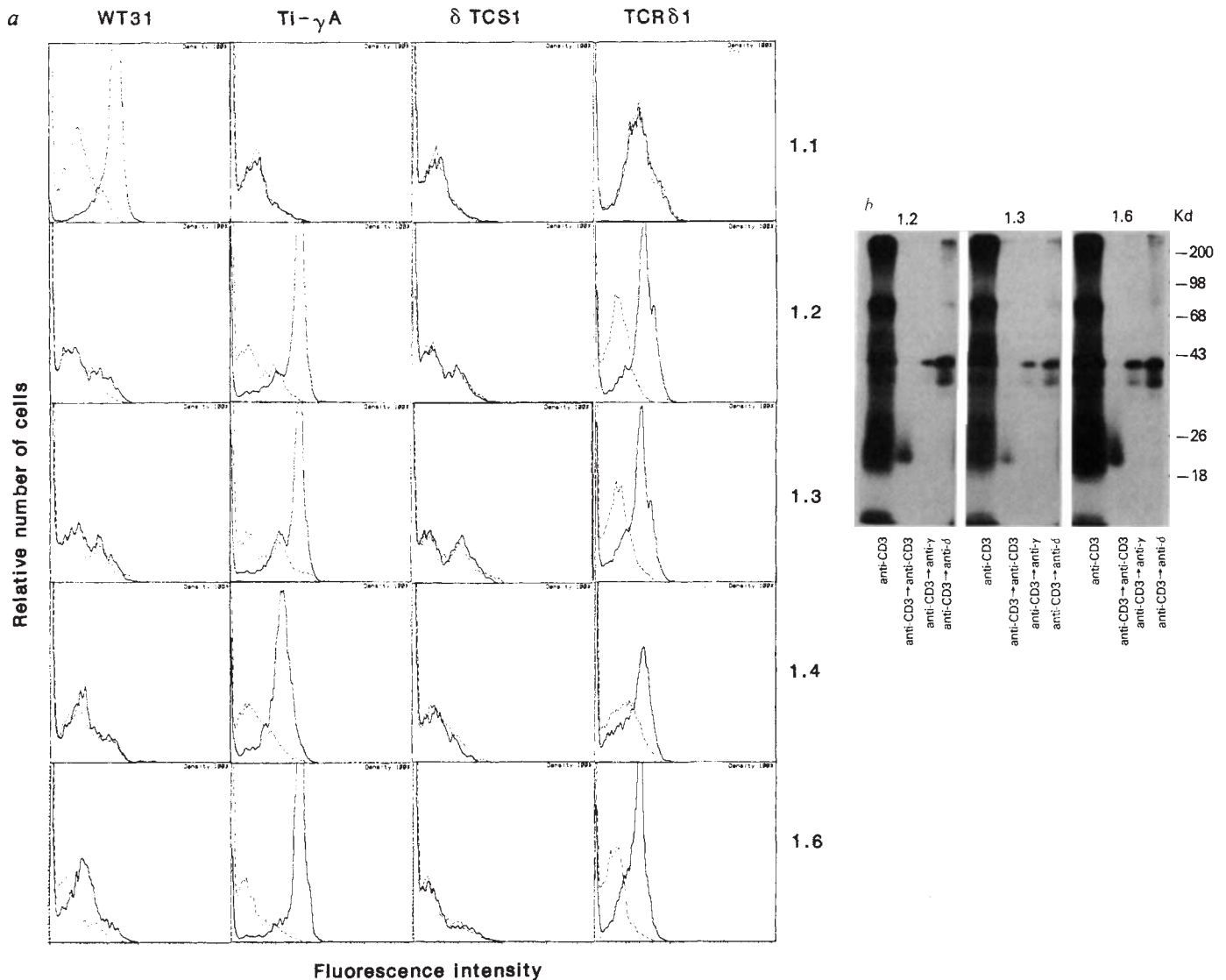


FIG. 2 Surface expression of TCR chains by the clones. *a*, The CD4⁺ clone 1.1 and the four double-negative clones 1.2–1.6 were analysed by indirect immunofluorescence using the following antibodies: WT31 (Becton-Dickinson), specific for α/β framework determinant; Ti- γ A, specific for TCR γ -chain¹⁴; δ -TCS1 (T Cell Sciences), specific for TCR δ -chain, and TCR δ 1 (prepared by Dr M. Brenner²² and provided by Dr L. Lanier), specific for TCR δ -chain. Fluorescence of test antibodies (solid line), superimposed on fluorescence intensity obtained with a control antibody (broken line) is shown. *b*, Immunoprecipitation analysis. Cells from double-negative clones 1.2, 1.3 and 1.6 were surface-iodinated and lysed in digitonin lysis buffer as previously described²³. After preclearing, immunoprecipitations were carried out using an anti-human CD3 δ serum²⁴ (provided by A. Weiss). The

precipitates were washed and TCR-CD3 complexes eluted by incubating for 10 min at 80 °C in 200 μ l of a 1% SDS solution. After centrifuging the PAA beads, the supernatant was removed and divided in four equal aliquots. One of these was directly analysed on SDS-PAGE under reducing conditions (lane 1). NP40 (200 μ l, 1.5%) was added to the other aliquots and these were subsequently used for reprecipitation with anti-CD3- δ serum (lane 2), anti-CD3- γ serum (lane 3) or with anti-TCR- δ serum (lane 4) before analysis on SDS-PAGE under reducing conditions. Molecular weight markers are indicated on the right. The anti-human CD3- δ and TCR γ -chain sera were made against peptides corresponding to the C-terminal 14 and 7 residues of these chains respectively^{23,25}.

TABLE 2 CD4⁺ and double negative clones proliferate in response to AP-MT

T cell clone	Phenotype	AP-MT	Proliferative response (stimulation index $\pm$ s.d.)		
			MBP	P64	PHA
1.1	CD4 ⁺ , α/β	12.3 $\pm$ 0.1	ND	ND	15.8 $\pm$ 0.1
1.2	DN, γ/δ	9.4 $\pm$ 0.1	2.6 $\pm$ 0.1	ND	39.4 $\pm$ 1.1
1.3	DN, γ/δ	12.0 $\pm$ 0.2	1.2 $\pm$ 0.1	ND	33.6 $\pm$ 0.4
1.4	DN, γ/δ	4.1 $\pm$ 0.1	1.0 $\pm$ 0.0	ND	7.5 $\pm$ 0.1
1.6	DN, γ/δ	7.4 $\pm$ 0.1	0.9 $\pm$ 0.2	5.4 $\pm$ 0.1	11.6 $\pm$ 0.2
BF1	DN, γ/δ	1.5 $\pm$ 0.1	ND	ND	16.9 $\pm$ 0.3
8.1	CD8 ⁺ , α/β	1.3 $\pm$ 0.2	ND	ND	37.7 $\pm$ 0.5

Proliferation assays were carried out as described in the legend to Fig. 1 using 15×10^3 T cells and 75×10^3 irradiated ($3,000$ R) autologous peripheral blood mononuclear cells per well in the presence or absence of AP-MT ($10 \mu\text{g ml}^{-1}$) guinea pig myelin basic protein (MBP, $20 \mu\text{g ml}^{-1}$, provided by Dr L. Steinman), a biochemically purified 64 K heat shock protein of mycobacterium bovis BCG (P64, $1 \mu\text{g ml}^{-1}$, prepared as described²⁰) or phytohaemagglutinin (PHA; $0.25 \mu\text{g ml}^{-1}$). Cultures were pulsed with [^3H] thymidine at 54 h and harvested at 72 h. A representative experiment, one of at least five repetitions is shown for each clone. Mean c.p.m. $\pm$ s.d. obtained without antigen were $1,238 \pm 242$, 364 ± 142 , 399 ± 57 , $1,513 \pm 437$ and 624 ± 115 for clones 1.1, 1.2, 1.3, 1.4, and 1.6 respectively. Control clones of the double-negative, γ/δ (BF1) or CD8⁺ α/β (8.1) phenotype were derived from the peripheral blood of a normal individual as described¹⁶ and were provided by Drs A. Rivas and E. Engleman. Their mean c.p.m. $\pm$ s.d. without antigen were 138 ± 7 and 141 ± 120 respectively. ND, not determined.

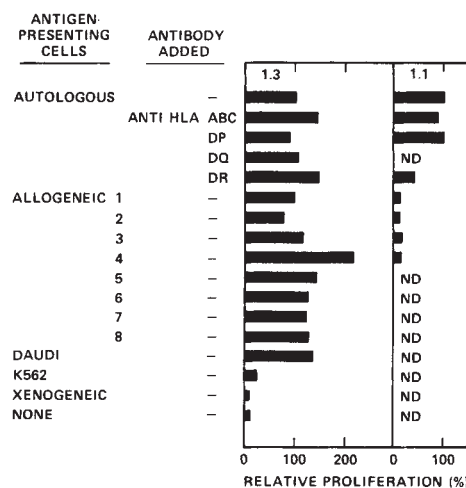


FIG. 3 Proliferation of double-negative clones to AP-MT is MHC non-restricted. The double-negative clones, represented here by clone 1.3 (left side) proliferated in the presence of both autologous and allogeneic antigen-presenting cells obtained from randomly selected nonrelated individuals. HLA typing was disparate among the allogeneic and autologous cells (not shown). Proliferation of the double-negative clones to AP-MT could not be blocked by anti-class I or class II antibodies.

METHODS. Proliferation assays were as described in the legend to Table 2. Autologous or allogeneic peripheral blood mononuclear cells, or xenogeneic (Lewis rat spleen) cells, or Daudi and K562 cells were used as a source of irradiated antigen-presenting cells. Experiments were done in the presence and absence of these antigen-presenting cells, and in the presence or absence of AP-MT ($10 \mu\text{g ml}^{-1}$). Anti-HLA monoclonal antibodies were added as described²⁶. These included anti-HLA ABC (W6/32, Accurate), anti-HLA DP (B7/21, B.D.), anti-HLA DQ (Leu 10, B.D.), or anti-HLA DR (L 243, B.D.). Results are presented as relative proliferative response (stimulation index obtained with AP-MT in the specified test conditions divided by the stimulation index obtained with AP-MT in the presence of autologous antigen-presenting cells, $\times 100$).

MT could be found in the absence of antigen-presenting cells, or when xenogeneic cells were used (Fig. 3). Taken together, these results indicate that recognition of AP-MT by the double-negative clones was not restricted by MHC and did not require interaction with class I MHC antigens. Recognition may, however, require presentation by a nonpolymorphic determinant of class II MHC gene product, or by different cell-surface antigens not expressed on K562 cells. This issue may be addressed by the use of cell lines transfected with class II MHC encoding genes.

T cells bearing the double negative phenotype have been implicated in a variety of murine autoimmune conditions³⁻¹⁶. For example, L3T4⁺, Lyt-2⁺ T cells were shown to be capable

of helping B cells to produce IgG antibodies to DNA in three systemic lupus erythematosus mouse strains⁵. Moreover, double-negative splenic T cells from normal mice were found to be capable of breaking oral tolerance¹⁷ and to serve as precursors of autoreactive T cells⁶. Non-malignant, human, double negative, TCR γ/δ -bearing cells have been isolated from the peripheral blood of a normal adult¹⁸, a fetus¹⁹ and an immunodeficient patient¹, but as yet there is no evidence to implicate them in the pathogenesis of autoimmune diseases.

Our data show that double-negative γ/δ cells can proliferate in response to a specific soluble antigen. The clones we describe were isolated from an autoimmune inflammatory site, and as such may be pathogenically relevant. □

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A point mutation in the *neu* oncogene mimics ligand induction of receptor aggregation

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THE rat *neu* gene, which encodes a protein closely related to the epidermal growth factor receptor¹, is a proto-oncogene that can be converted into an oncogene by a point mutation. Both genes encode proteins with a relative molecular mass of 185,000 but the question of why the *neu* gene product, p185^{neu}, is oncogenic, whereas the product of *c-neu*, p185^{c-neu}, is not, remains unanswered. The proteins have several features common to the family of tyrosine kinase growth-factor receptors, including cysteine-rich external domains, a hydrophobic transmembrane region and a cytoplasmic tyrosine kinase domain²⁻⁴. The oncogenic p185^{neu} differs from p185^{c-neu} by an amino-acid substitution in the transmembrane region of the glycoprotein⁵: this replacement of valine by glutamic acid at position 664 induces increased intrinsic tyrosine kinase activity which is associated with transformation^{6,7}. Many glycoproteins with charged amino acids in the transmembrane region exist as multimeric complexes at the plasma membrane. We have therefore investigated the association state of both products of the *neu* gene and show that the oncoprotein p185^{neu} is organized at the plasma membrane primarily in an aggregated form, but that p185^{c-neu} is not. Induction of an aggregated state may mimic aspects of ligand-induced receptor aggregation resulting in enzymatic activation that leads to cellular transformation.

We analysed the aggregation states of the proto-oncogenic and oncogenic forms of the *neu*-encoded protein (Neu). B104-1-1 is an NIH 3T3 transfectant cell line that expresses the oncogenic p185^{neu} and displays growth characteristics of a transformed cell line, including the ability to form tumours in nude mice and colonies in soft agar. A second NIH 3T3 transfectant cell line, DHFR/G8, expresses the proto-oncogenic p185^{c-neu} and does not have the characteristics of transformed cells. The oncogenic phenotype is a property intrinsic to the structural and enzymatic features of the p185^{neu} molecule³⁻⁷.

We used cross-linking to study the possible association of Neu proteins on the cell surface (Fig. 1). Both the proto-oncogenic and oncogenic proteins cross-linked with other proteins, as deduced by the appearance of a higher-molecular weight product resolving at about 360,000–400,000 (360–400K). In replicate assays we found that >70% of oncogenic p185^{neu} exists as an aggregated cross-linkable complex, whereas only 20–30% of proto-oncogenic p185^{c-neu} can be cross-linked under identical conditions (Fig. 1). A high-molecular weight form of p185^{neu} was also observed in the neuroblastoma-derived cell line B104 which also expresses the oncogenic Neu protein (data not shown).

We observed high-molecular weight proteins in the absence of cross-linking reagents added under non-reducing conditions (Fig. 1). We therefore compared proteins prepared from B104-1-1 and DHFR/G8 cells on low-percentage polyacrylamide gels to resolve those of higher molecular weight (Fig. 2). We found that under non-reducing conditions the proto-oncogenic protein migrated faster than the oncogenic protein, and that in the lane containing the oncogenic p185^{neu} a second higher-molecular weight band was evident at 360–390 K, which is about double the molecular weight of the monomer p185 protein. This could indicate either that the oncogenic p185^{neu} is covalently attached

to a second protein or that the larger protein represents a high-molecular weight form of p185^{neu}.

To test the importance of disulphide linkages in the high-molecular weight forms of p185^{neu}, we immunoprecipitated the proto-oncogenic and oncogenic proteins, then reduced the disulphide bonds in 4% SDS-polyacrylamide gels with a gradient of 2-mercaptoethanol⁸ (Fig. 3). The p185^{neu} species undergo different transitions during reduction: p185^{c-neu} gives at least three forms of different molecular weights which range from ~170K (non-reduced) to 182K (reduced). There is no evidence for association with other molecules because the intensity of the different bands is constant during the transition. The low-molecular weight oncogenic p185^{neu}, however, remains relatively constant at 185K, but the high-molecular weight species disappears dramatically during reduction, with a concurrent increase in the intensity of the low-molecular weight oncogenic p185^{neu} band. The slightly increased molecular weight of oncogenic p185^{neu} may be due to differences in phosphorylation¹. We have also analysed the aggregation states of the human epidermal growth factor receptor and the product of the human *neu* proto-oncogene on gradient reducing gels. Neither protein is charged in its transmembrane domain. As expected, their electrophoretic resolution patterns are similar to proto-oncogenic p185^{c-neu} (data not shown). Together our observations indicate that the Neu oncoprotein can exist as a monomer or as an aggregated homodimer.

It is possible that the point mutation in the transmembrane region resulting in the amino-acid change of the oncogenic

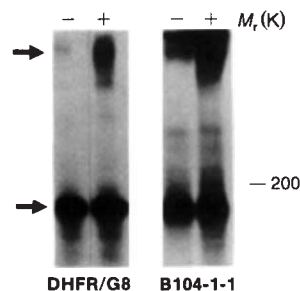


FIG. 1 Cross-linking analysis of p185^{neu} oncogenic and proto-oncogenic phosphoproteins. The homobifunctional cross-linking reagent dithiobis(succinimidyl propionate) (DSP), with an approximate cross-linking radius of 12 Å, was used to cross-link spatially related polypeptides on the cell surface²¹. B104-1-1 or DHFR/G8 cells were incubated for 30 min in DSP as described²². Cells were then lysed, immunoprecipitated and separated by SDS-PAGE in the absence of reducing agents. Arrows indicate high-molecular weight forms of p185^{neu}.

METHODS. Cell labelling and lysis were as described^{19,23} but with the following modifications. ³²P-labelled cells were incubated with (+) or without (-) DSP cross-linking reagent in 800 µl PBS containing 1 mM MgCl₂ and 0.02% sodium azide, pH 7.4. DSP at 10 mg ml⁻¹ in dimethylsulphoxide was added to the cells to a final concentration of 10 µg ml⁻¹ and allowed to react at 23 °C, with occasional rocking of the plates, for 30 min on ice⁸. Viability was >95%, as confirmed by trypan-blue exclusion before solubilization. Plates were extensively washed for 30 min in Tris-buffered saline before lysis in PI/RIPA buffer (1% NP40, 0.1% deoxycholate, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.4, 1 mM PMSF, 2 mM EDTA, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 400 µM sodium orthovanadate, 10 mM iodoacetamide (to prevent acetylation) and 1 mM ATP). Pre-cleared supernatants were immunoprecipitated with monoclonal antibody against rat p185^{neu} (7.16.4) (refs 19 and 23). The immunoprecipitated antigen receptor was eluted from the antibody-bead complex with 25 µl SDS-sample elution buffer (2% SDS, 20% glycerol, 50 mM Tris-HCl, pH 6.8, with or without 5% 2-mercaptoethanol) and boiled for 3 min. Molecular weight standards (Pharmacia) were visualized with Coomassie blue dye and included in all slab gels. Samples were resolved by electrophoresis on 4%–7.5% gradient SDS-polyacrylamide gels. Dried gels were exposed to XAR film at -80 °C.

p185^{neu} activates the molecule, and that receptor clustering is a secondary event related to receptor activation and increased enzyme function. Alternatively, the mutation itself might not activate the Neu oncoprotein, activation being a result of the single amino-acid substitution at position 664, so permitting the aggregation of receptor proteins. This argument would implicate specific regions of growth factor receptors, such as the transmembrane and possibly the cysteine-rich domains, in the protein-protein associations that lead to activation of the enzymatic domains. Cysteine-rich domains may be involved in the non-covalent dimerization of DNA-binding proteins⁹, but their function in growth-factor receptors is unknown. Our analysis of transmembrane residues from a variety of plasma membrane proteins demonstrates that many aggregated surface molecules

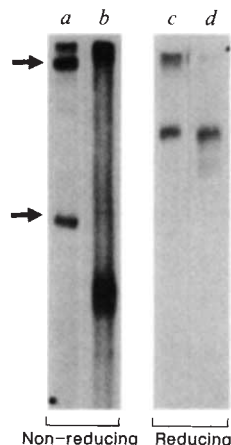


FIG. 2 SDS-PAGE analysis of radiolabelled immunoprecipitates of DHFR/G8 and B104-1-1 by monoclonal antibody 7.16.4. *a* and *c*, B104-1-1 immunoprecipitated with 7.16.4 under non-reducing conditions (in the absence of 2-mercaptoethanol) in 4% acrylamide, 0.17% bisacrylamide gels and under reducing conditions (5% in the presence of 2-mercaptoethanol) in 7.5% acrylamide, 0.17% bisacrylamide gel, respectively. *b* and *d*, DHFR/G8 immunoprecipitated with 7.16.4 under non-reducing or reducing conditions as for *a* and *c* respectively. Arrows indicate different p185^{neu} forms.

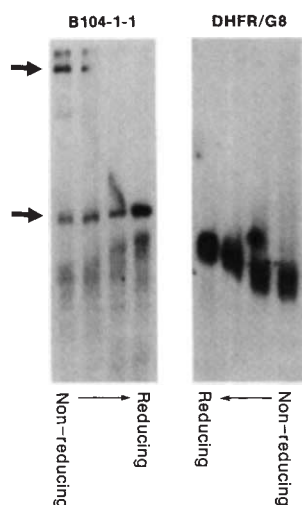


FIG. 3 SDS-PAGE incorporating a 2-mercaptoethanol gradient. The same number of counts of proto-oncogenic DHFR/G8 and oncogenic B104-1-1 p185^{neu} immunoprecipitates were loaded onto four adjacent lanes of a 4% polyacrylamide gel. Reducing lanes were prepared with 2-mercaptoethanol: the gel was run overnight at low voltage to allow diffusion of reducing agent added to the interior lanes towards the exterior lanes⁸.

contain charged amino acids¹⁰⁻¹³. An example of such an association is found in the T-cell receptor-associated protein CD3 zeta¹⁴, in which there is a single negatively charged aspartic acid in the transmembrane region and the polypeptide exists as a homodimer disulphide-linked surface protein.

Activation of cellular functions through aggregation of a receptor protein has been described¹⁵: when incubated with epidermal growth factor (EGF), the EGF receptor changes from a monomer to a dimer¹⁶⁻¹⁸. The activated EGF receptor is subsequently down-modulated from the cell surface. But aggregation by itself cannot be responsible for internalization and receptor down-modulation, because aggregated p185^{neu} is expressed on the cell surface¹⁹. Recently, other point mutations have been shown to induce the oncogenic phenotype of p185^{neu} (ref. 20) but the aggregation state of the encoded proteins has not been determined. The induction of an associated or aggregated receptor phenotype by substitution of a single charged amino acid in the transmembrane region demonstrates a novel mechanism of oncogenesis warranting further investigation. □

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Retinoic acid regulates growth hormone gene expression

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VITAMIN A is required for normal growth and development, and retinoic acid (RA) may be the active metabolite in this process¹. Recent evidence indicates that RA acts through binding to a nuclear receptor²⁻⁵ which belongs to the steroid/thyroid hormone receptor superfamily⁶. The receptors seem to associate with hormone-response elements in the target genes resulting in the activation (or inhibition) of transcription⁶. Although no interaction of RA-receptor complex with specific DNA sequences has yet been reported, the homology of the different receptors suggests their mechanisms of action are similar. We therefore examined whether the

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effects of RA on growth could be related to changes in the expression of the growth hormone gene which is known to be transcriptionally regulated by both thyroid and glucocorticoid hormones⁷. Our results show that RA controls growth hormone production in pituitary GH1 cells and that its effect is synergistic with that caused by these hormones.

In GH1 cells thyroid hormones stimulate growth hormone (GH) production, and glucocorticoids potentiate the action of thyroid hormones⁸. This is seen in Fig. 1a which shows the stimulatory effects of 0.05 nM triiodothyronine (T3) and 50 nM dexamethasone (Dx) in the presence or absence of 1 μ M RA; within 48 h, RA by itself produced an increase in GH production which ranged between three- and five-fold that of control levels. In the experiment illustrated (Fig. 1a), 1 μ M RA elicited an increase similar to that produced by T3 at a concentration (0.05 nM) that produces less than a half-maximal response⁸. However, RA greatly potentiated the action of this low dose of T3 and in the presence of both compounds, GH levels were at least 15-fold higher than in non-stimulated cells. A similar increase occurred when RA was combined with Dx, whereas the steroid alone induced no detectable response. The combination of RA plus T3 or RA plus Dx was more effective than the combination of T3 plus Dx, and RA produced further stimulation, more than 30 times that of control levels, when T3 and Dx were present together. With different concentrations of T3, RA was a more important regulator than Dx, and a clear synergistic effect of the three agents was found under all conditions (see Fig. 1b).

In a dose-response study for GH induction by RA and T3, with and without Dx, RA alone (0.001–10 μ M) stimulated a dose-dependent induction of GH production (Fig. 1c). The effect of concentrations lower than 0.1 μ M was modest, whereas between this dose and 10 μ M RA, GH levels rose abruptly. This dose-response is rather unexpected for an RA receptor-mediated effect: the effective dose (50%) (ED₅₀) for chimaeric RA receptors to activate a reporter gene ranges from 1–10 nM^{3,4}, which agrees with the concentrations of RA that mediate morphogenic effects of the retinoid⁹, promote differentiation and influence the transcription of specific genes in cultured cells^{10,11}.

Interestingly, in the presence of 0.1 nM T3, the dose-response curve for RA to induce the GH response was shifted to the left by at least a factor of 1,000, with the ED₅₀ changing from more than 1 μ M in the absence of thyroid hormone, to less than 0.01 μ M in the presence of 0.1 nM T3. Moreover, with T3 at a concentration (5 nM) that saturates the triiodothyronine receptor and fully induces GH⁸, 0.001 μ M RA elicited an almost maximal effect. The pattern of response to RA in the presence of Dx was similar to that found with RA alone. Dx, therefore, seems to potentiate the action of RA without greatly altering the RA dose-response characteristics. On the other hand, when Dx was combined with T3, the response to RA was again found at low concentrations, and the ED₅₀ was shifted from micromolar to nanomolar concentrations.

The changes in GH production parallel those found in GH messenger RNA levels (Fig. 2); RA therefore acts at the pre-translational level to increase the rate of accumulation of mRNA. RA induced a two- to threefold increase in GH mRNA levels, and the response of the cells incubated with T3 plus RA or Dx plus RA was fivefold greater than for T3 or Dx alone (Fig. 2a). T3 plus Dx induced a fourfold increase in GH mRNA, and this was further increased by a factor of 3.5 in the presence of RA. In the absence of thyroid hormones, the effect of 5 nM RA was negligible; although the same concentration of the retinoid produced a sixfold increase of GH mRNA in the presence of 0.1 nM T3 (Fig. 2b). In accord with the known potency of retinoids^{3,4}, retinol and retinyl acetate were also effective in inducing GH mRNA levels synergistically with T3 and Dx, although at higher concentrations than RA (Fig. 2c).

These results indicate that the rate of GH production is directly related to GH mRNA levels, and that RA acts synergisti-

cally with thyroid hormones and glucocorticoids to stimulate GH mRNA accumulation in GH1 cells. RA stimulation of GH gene expression could occur by prolongation of the mRNA half-life and/or by increasing the rate of GH gene transcription and mRNA synthesis. In cultured GH-producing cells, the half-life of GH mRNA is ~40 h and is not altered by T3 or glucocorticoids⁷. The kinetics of accumulation of GH mRNA by RA, illustrated in Fig. 3, suggest that the effect of the retinoid must be at least partially due to transcriptional regulation, as stabilization of a mRNA with such a long half-life cannot account for the observed time course. To determine whether RA regulates the gene transcriptionally, we conducted nuclear 'run-on' experiments after incubation with RA alone or in combination with T3 plus Dx. Figure 4a shows that GH gene transcription rates were stimulated by a factor of 4.9 after incubation for 4 h with

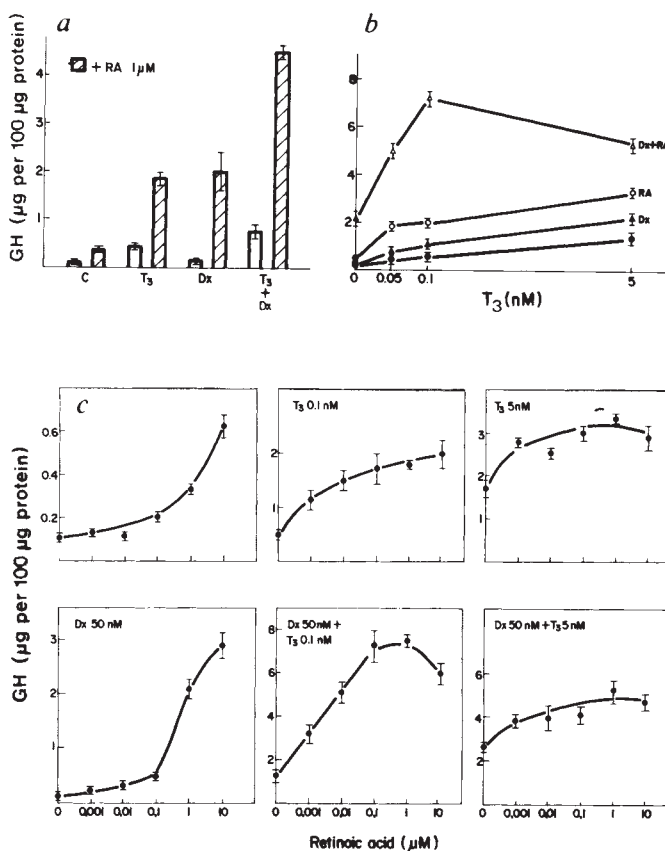


FIG. 1 Influence of retinoic acid on the induction of GH production by T3 and dexamethasone. *a*, GH1 cells were incubated in medium alone (C), with 0.05 nM triiodothyronine (T3), 50 nM dexamethasone (Dx) or the combination of both (T3 + Dx) in the absence (open bars) or presence (stippled bars) of 1 μ M RA. *b*, The cells were incubated with increasing concentrations of T3 in the presence or absence of 50 nM Dx and/or 1 μ M RA. *c*, Dose-response induction of growth hormone by retinoic acid.

METHODS. GH1 cells were grown as previously described¹² with RPMI 1640 medium containing 10% horse serum and 2.5% fetal calf serum. The medium was exchanged 48 h before the experiments with medium containing 10% thyroid hormone and glucocorticoid-depleted newborn calf serum¹². The cultures were incubated for a further 48 h in the presence of different compounds, and growth hormone was quantified in the medium by radioimmunoassay, using the reagents provided by the NIAMDD, NIH. In GH1 cells the accumulation of GH in the medium is an accurate assessment of the rate of GH synthesis as the hormone does not accumulate intracellularly and it is stable in the medium⁸. Rates of production of GH are expressed as μ g hormone produced per 100 μ g of cell protein, determined by the method of Lowry *et al.*¹⁸. In *c*, the cells were incubated with increasing concentrations of retinoic acid. Where indicated, T3 and Dx were used in combination with retinoic acid. Note that the different ordinates correspond to the varied stimulation of GH production.

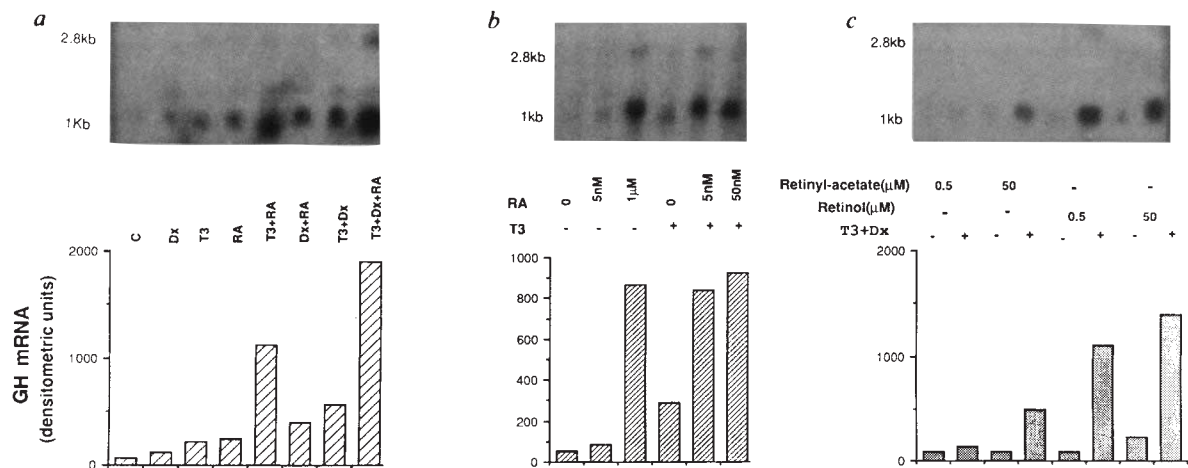


FIG. 2 Induction of GH mRNA by retinoic acid. Northern blot analysis was performed with total RNA (60 µg) extracted from GH1 cells treated for 42 h with: a, 1 µM retinoic acid (RA), 0.1 nM triiodothyronine (T3) or 50 nM dexamethasone (Dx); b, RA, 0, 5 nM and 1 µM in the presence or absence of 0.1 nM T3; and c retinylacetate, retinol or RA in the presence or absence of 0.1 nM T3 plus 50 nM Dx (T3+Dx). Quantitative analysis of the densitometric scans of the autoradiographs are presented in the corresponding lower panels.

METHODS. Total RNA was isolated from GH1 cells with guanidine isothiocyanate¹⁹, separated on 1% agarose-formaldehyde gels, transferred to nylon membranes (Nytran) and hybridized with a 802-bp *Hind*III fragment from p-rGH-1, a rat recombinant (r) rGH cDNA-bearing plasmid²⁰. This insert represents more than 95% of the mature rat GH mRNA. The probe was labelled with ³²P using random oligonucleotides as primers for the Klenow

subunit of DNA polymerase I (specific activity, 1×10^8 c.p.m. µg⁻¹). The blots were pre-hybridized and hybridized under the conditions recommended by the manufacturer (Schleicher & Schuell). The filters were autoradiographed overnight at -70 °C, and GH mRNA was quantified by scanning densitometry of the 1-kilobase (kb) band corresponding to the mature GH mRNA in the autoradiographs. After quantification, the membranes were washed and re-hybridized with a [³²P]β-actin probe. The levels of β-actin mRNA did not vary with the different treatments (not shown). The size of the RNAs was determined by comparison with a standard RNA ladder (BRL). Similar results were obtained by quantification of dot blots in which the preparations of RNA were diluted sequentially, fixed with formaldehyde, immobilized in nylon membranes and hybridized with the [³²P]p-rGH-1 fragment under the same conditions.

1 µM RA. The combination of T3 (0.1 nM) plus Dx (50 nM) induced a 2.8-fold increase, and this response was further increased to 9.5-fold in cells incubated with RA.

These observations support the idea that the RA-receptor complexes interact with DNA sequences in the GH gene to elicit direct control of transcription. Moreover, the RA-response element is located in the 5'-flanking DNA of the rat GH gene (Fig. 4b). When GH1 cells were transiently transfected by electroporation^{12,13} with a reporter plasmid containing 1,800 base pairs of the rat GH promoter ligated to the chloramphenicol acetyl transferase (CAT) gene, 1 µM RA stimulated CAT activity ninefold, 0.1 nM T3 induced a fivefold stimulation, and the combination of both produced a further stimulation (36-fold). RA and thyroid hormone therefore act independently and synergistically to regulate the expression of the gene.

Although other genes have been shown to be transcriptionally regulated by RA¹⁴, promoter regions of these genes were not available. The GH gene provides an excellent model for the determination of the molecular mechanisms of RA action because the *cis*-acting DNA sequences and *trans*-acting regulatory proteins which are essential for cell-specific (basal) expression and transcriptional stimulation of the gene, have been extensively studied^{12,15}.

Unlike the synergistic effect of RA and glucocorticoids which is not accompanied by a change of the dose-response curve, much lower concentrations of RA are required in the presence than in the absence of thyroid hormones. Significantly, the RA receptors, which along with other nuclear receptors are thought to have evolved from a common ancestor⁶, are most closely related to the thyroid hormone receptors. Our observations also show that the regulation of GH gene expression by T3, RA and glucocorticoid receptors is an evolutionarily conserved function, thus suggesting that their similarity is not only structural, but also functional. The *cis*-active elements that mediate the positive transcriptional effects of steroids and thyroid hormones exhibit

a high degree of sequence similarity¹⁵, and the response elements for different steroids can be closely localized in the promoter regions of regulated genes, or are even functionally indistinguishable¹⁶. By analogy, it is reasonable to assume that the RA and T3 receptors are a part of a functional 'enhancer-like unit' in the promoter of the GH gene. After submission of this paper, Umesonon *et al.* described how RA can activate the expression of an artificial gene through the thyroid hormone response element present in the rat GH gene, thus implying that RA and thyroid hormones could regulate overlapping gene networks¹⁷. Our data show that this is indeed the case, and that the natural GH promoter is regulated by both hormones. Taken together,

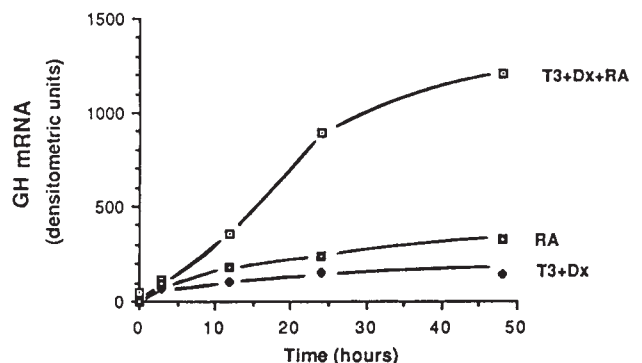


FIG. 3 Kinetics of GH mRNA accumulation in response to retinoic acid. The effect of RA on both basal and T3+Dx induced mRNA levels was already detectable after 3 h and was increased by a factor of 3-4 after 12 h. GH mRNA levels were maximal after 48-72 h, and longer incubation times did not produce further increases (data not shown). GH1 cells were incubated as indicated with 0.1 nM triiodothyronine (T3) and 50 nM dexamethasone (Dx) in the presence or absence of 1 µM retinoic acid (RA). GH mRNA levels were analysed by dot-blot and quantified as described in Fig. 2.

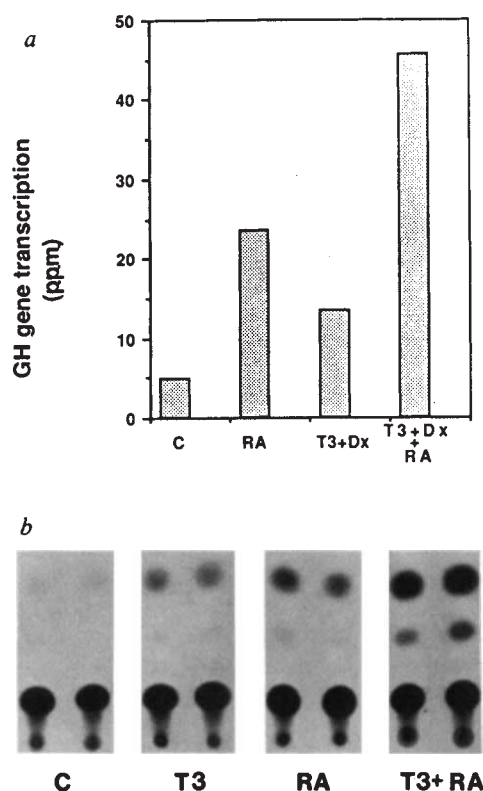


FIG. 4 *a*, Transcriptional regulation of the GH gene by retinoic acid (RA). GH1 cells were incubated with triiodothyronine (T3) (0.1 nM), dexamethasone (Dx) (50 nM) and/or with retinoic acid (RA) (1 μ M). The GH gene transcription rates were determined after 4 h of incubation with these compounds. *b*, Effect of RA and T3 on transient gene expression of a pGH-CAT plasmid. The study was performed using a chimaeric plasmid in which 1,800 base pairs of 5'-flanking DNA of the rat GH gene were ligated upstream of the CAT gene (p-GH-CAT(-1800))¹³. The cells were transfected with the plasmid and CAT activity was determined after 48 h of incubation with 0.1 nM T3, 1 μ M RA or a combination of both.

METHODS. *a*, For the 'run-on' assays, the nuclei were isolated and incubated *in vitro* with 250 μ Ci [α -³²P]UTP. The [³²P]RNA was isolated, purified and hybridized to nylon filters containing pRGH1 or pBR322 plasmid DNA as described by Yaffe and Samuels⁷. The results are expressed as p.p.m. of the total [³²P]RNA input in the hybridizations. *b*, The cells were transfected by electroporation with 10 μ g plasmid, and CAT assays were performed with 50 μ g cell lysate protein as previously described^{2,13}. After autoradiography, the acetylated and non-acetylated [¹⁴C]chloramphenicol was quantitated. In control cells the acetylated forms represented 0.2% of the total label and this value increased to 1.9%, 1.0% and 7.2% in cells incubated with RA, T3 and T3+RA, respectively.

these results suggest that a common responsive element could mediate the regulation of GH gene expression by T3 and RA.

Our finding that RA affects GH production stresses the importance of retinoids for normal growth and development. As malnutrition and iodine (and thyroid hormone) deficiency are normally associated, our results could provide an explanation for the poor somatic growth that characterizes vitamin A deficiency. □

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In vitro correction of G·T mispairs to G·C pairs in nuclear extracts from human cells

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IN differentiated cells, only a specific subset of genes is expressed. Recently, several genes have been shown to be transcriptionally inactivated by methylation of cytosine residues, mainly within their promoter sequences¹⁻⁴. Spontaneous hydrolytic deamination of 5-methylcytosine to thymine⁵, which has been estimated to generate up to 12 G·T mismatched base pairs in the human genome per day^{6,7}, could have a deleterious effect on the expression of such genes. We recently reported that mammalian cells possess a specific repair pathway, which counteracts the mutagenic effects of this deamination by correcting G·T mismatches almost exclusively to G·C pairs^{8,9}. We show here that, in nuclear extracts from HeLa cells, this repair is mediated by excision of the aberrant thymidine monophosphate residue, followed by gap-filling to generate a G·C pair. We also provide preliminary evidence that the initial step of this process involves a DNA glycosylase.

In vitro mismatch repair experiments were carried out with synthetic 90-mer substrates. The single G·T mismatch was situated within the sequence $\begin{matrix} \text{GTCTGAC} \\ \text{CAGTTG} \end{matrix}$. In the absence of the mismatch, this sequence can be cleaved by the restriction endonucleases *AccI*, *HincII* and *SalI* (Fig. 1*a*). The G·T mismatch renders the duplex resistant to hydrolysis by *SalI* (ref. 10), although a small amount of nicking was observed in the upper strand of the 90-mer, which contains the *bona fide* restriction site (data not shown). The T-containing strand was not nicked by *SalI* under the conditions of the assay.

When the oligonucleotide duplex was incubated with the HeLa nuclear extract in the presence of ATP, Mg²⁺ and the four deoxynucleoside triphosphates (dNTPs), a proportion of it became susceptible to cleavage with *SalI* (Fig. 1*b*, lanes 1-3), presumably as the result of specific *in vitro* correction of the G·T mispair to a G·C pair. The treated duplex that was not subjected to *SalI* digestion remained mostly intact; only a small amount of nicking was detectable in the vicinity of the mispair (Fig. 1*b*, lanes 5-7). The possibility, however, that this G·T→G·C correction was in fact brought about by a fortuitous nick translation of the lower strand had first to be eliminated.

Incubation of the mismatched duplex with the nuclear extract in the absence of added ATP, Mg²⁺ and dNTPs, all cofactors required by DNA polymerases and ligases present in the extracts, was shown to result in the appearance of strand-specific nicks. These were detected almost exclusively in the lower, T-containing strand (Fig. 2*a*, lanes 5-7; compare with lanes 9-11). The nicks were shown to be positioned on either side of

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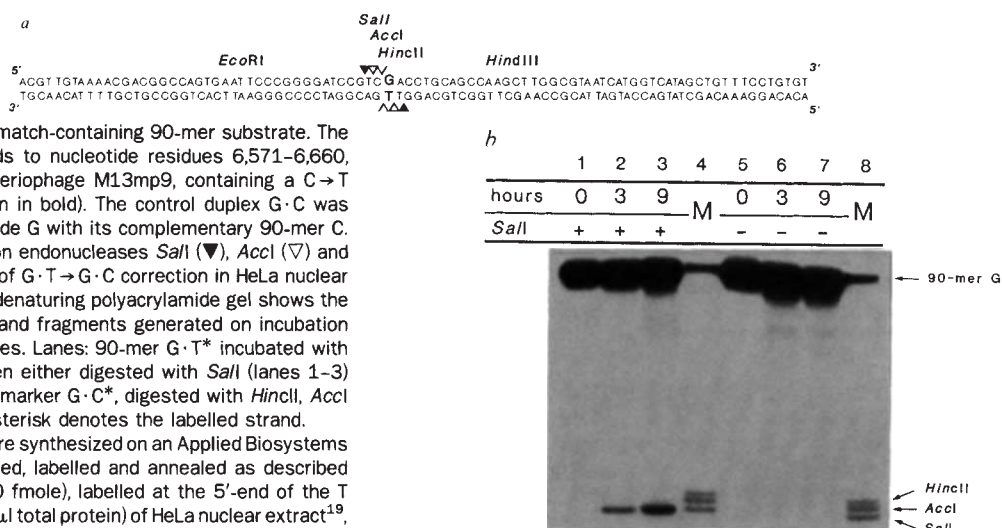


FIG. 1. **a**, Structure of the G·T mismatch-containing 90-mer substrate. The lower strand sequence corresponds to nucleotide residues 6,571–6,660, flanking the polylinker of the bacteriophage M13mp9, containing a C→T transition at position 6,620 (shown in bold). The control duplex G·C was obtained by annealing oligonucleotide G with its complementary 90-mer C. The cleavage sites of the restriction endonucleases *Sall* (▼), *AccI* (▽) and *HincII* (V) are indicated. **b**, Kinetics of G·T→G·C correction in HeLa nuclear extracts. This autoradiograph of a denaturing polyacrylamide gel shows the radioactively labelled 90-mer G·T and fragments generated on incubation with the extracts for the given times. Lanes: 90-mer G·T* incubated with the extracts for 0–9 hours and then either digested with *Sall* (lanes 1–3) or undigested (lanes 5–7); 90-mer marker G·C*, digested with *HincII*, *AccI* and *Sall* (M; lanes 4 and 8). The asterisk denotes the labelled strand. **METHODS.** The oligonucleotides were synthesized on an Applied Biosystems model 380A synthesizer and purified, labelled and annealed as described previously¹⁰. The G·T 90-mer (100 fmole), labelled at the 5'-end of the T strand, was incubated with 6 μ l (72 μ l total protein) of HeLa nuclear extract¹⁹, prepared as described by Krämer and Keller²⁰ in a final volume of 50 μ l. (All nuclear extracts tested, prepared by either method^{19,20}, could process G·T mispairs, irrespective of whether they were active in an mRNA-splicing assay). The reactions were carried out at 37 °C in buffer A (10x buffer A contains 250 mM HEPES-KOH, pH 7.9, 5 mM EDTA, 0.1 mM ZnCl₂, 5 mM DTT) supplemented with 40 mM KCl, 1 mM MgCl₂, 20 mM creatine phosphate, 50 μ M ATP and 100 μ M each dNTP. Aliquots (16 μ l) were withdrawn after the indicated lengths of time and frozen in dry ice. They were then treated with proteinase K (300 μ g ml⁻¹) for 30 min at 30 °C, extracted with phenol

then chloroform and ethanol-precipitated in the presence of 10 μ g transfer RNA carrier. The pellets were resuspended in 20 μ l of High Salt Buffer (Boehringer-Mannheim) and 10 μ l of each were digested with 4 units of *Sall* at 37 °C. After 90 min, all samples were reprecipitated with ethanol and the pellets were dissolved in formamide loading dye, denatured and applied to a 12% denaturing polyacrylamide gel. Electrophoresis was carried out at 30 mA until the xylene cyanol dye had migrated ~2/3 of the gel length.

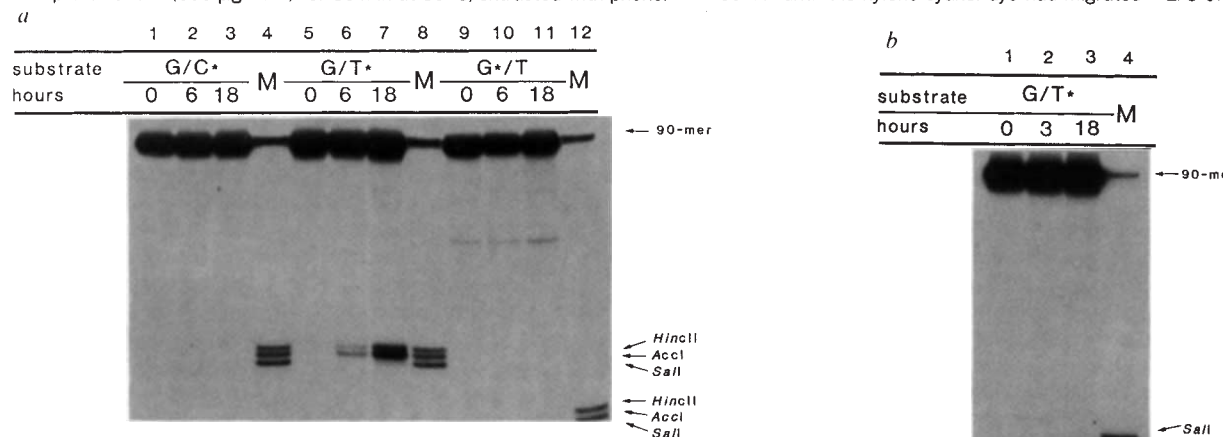
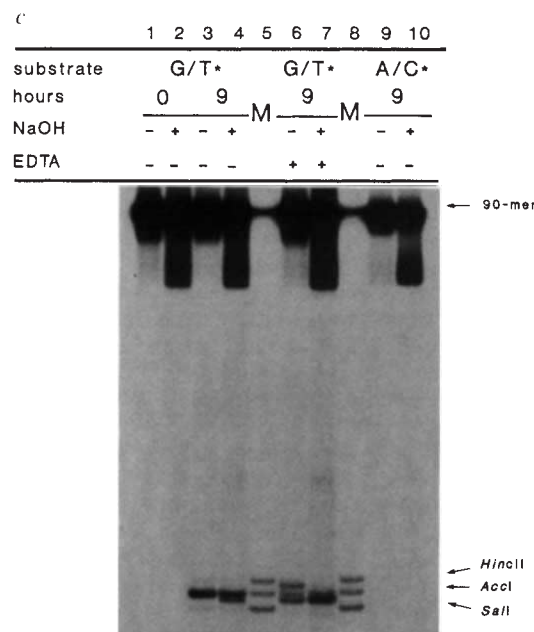


FIG. 2 Kinetics of mismatch-directed endonucleolytic cleavage of the 90-mer G·T in HeLa nuclear extracts. **a**, The duplexes G·C* (lanes 1–3), G·T* (lanes 5–7) and G*·T (lanes 9–11), labelled at the 5'-end on the strands indicated by asterisks, were incubated with the extracts for the indicated times. 90-mer G·C labelled at the 5'-end of oligonucleotide C (lanes 4 and 8), or G (lane 12), digested with *HincII*, *AccI* and *Sall*, was used as marker (M, see also Fig. 1b). The weak bands visible in lanes 9–11 are due to impurities in the labelled 90-mer G. The intensity of the upper band in lanes 6 and 7 varied from experiment to experiment (compare with c, lane 3). The band-pattern shown here was chosen because it clearly demonstrates the presence of two successive incisions. **b**, 90-mer duplex G·T*, labelled at the 3'-end of the oligonucleotide T, treated as in **a** (lanes 1–3). M, marker 90-mer G·C*, digested as above (lane 4). (Note the reversal of the band order, resulting from labelling of the oligonucleotides at their 3'-ends.) **c**, 90-mer duplexes G·T* (lanes 1–4 and 6 and 7) and A·C* (lanes 9 and 10), labelled at the 5'-ends of T, C strands were treated as in **a** but in the presence (+) or absence (–) of EDTA as indicated. M, marker as in **a**. The NaOH treatment (lanes 2, 4, 7 and 10) was carried out on samples isolated from the reaction mixture (see below).

METHODS. The duplexes were treated with the extracts as described in Fig. 1b, except that the reactions were carried out at 30 °C solely in buffer A for the indicated times. In **c**, EDTA was added to the reaction mixtures to a final concentration of 20 mM. The alkali treatment was performed as described¹¹: the duplexes were recovered from the reaction mixtures (see Fig. 1b) and resuspended in 5 μ l water, then half of each sample was mixed either with an equal volume of 0.2 M NaOH (lanes +) or water (lanes –) and heated at 90 °C for 30 min. Electrophoresis was carried out using 40 cm, 12% polyacrylamide denaturing gels until the xylene cyanol marker dye migrated 2/3 of the gel length.



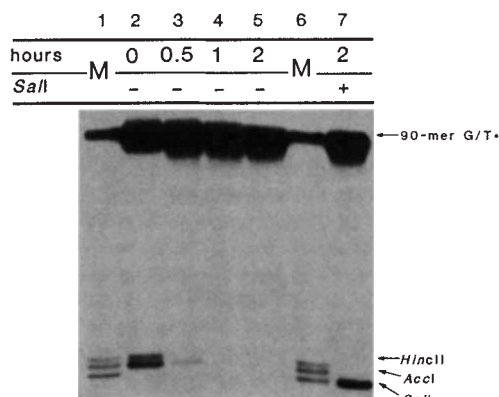


FIG. 3 Kinetics of repair of the nicked duplex G·T*, 5'-labelled on the T strand: lanes 2-5, nicked 90-mer treated with the nuclear extracts for 0-2 hours in the presence of ATP, Mg²⁺ and dNTPs; lane 7, 90-mer treated as above for 2 hours and digested with *SalI*; lanes 1 and 6, 90-mer G·C* digested with *HincII*, *AccI* and *SalI*. The asterisk denotes the 5'-labelled strand.

METHODS. The 90-mer G·T* was incubated with the extract as described in Fig. 2. After 16 hours, the reaction mixture was diluted with 25 μ l 10x buffer B (200 mM HEPES-KOH pH 7.9, 1 mM EDTA, 2.5 mM DTT, 200 mM KCl, 50 mM MgCl₂, 100 μ M each dNTP, 200 mM creatine phosphate, 30 mM ATP), 30 μ l of the nuclear extract and water to give a total volume of 250 μ l. Incubation was carried out at 37 °C and 60 μ l aliquots, withdrawn at the indicated times, were treated as described in Fig. 1b.

the mispaired thymidine residue by virtue of the bands co-migrating with the *HincII*- and *AccI*-cleaved 90-mer markers.

Treatment of the G·T duplex labelled at the 3'-end of the T strand gave rise to only a single labelled fragment, comigrating with the *HincII* marker (Fig. 2b). Taken together, these results demonstrate that the T strand was initially cleaved immediately 3' from the mispaired thymidine, and that this was probably followed by a rapid 3' $\rightarrow$ 5' excision of the aberrant TMP residue. As the G strand of the mismatched duplex (Fig. 2a, lanes 9-11), as well as both strands of 90-mer duplex containing an A/C mispair instead of a G·T pair (Fig. 2c, lane 9 and data not shown), remained intact under these experimental conditions, it can be concluded that the observed nicking is not due to a non-specific endonucleolytic processing of DNA containing a helical distortion, but rather to a specific incision targeted to the mispaired thymine of a G·T mismatch.

The above data show that only the mispaired TMP was removed from the 90-mer G·T duplex in HeLa nuclear extracts. The strand-specificity and directionality of this process correlate extremely well with those observed *in vivo*^{8,9}. Interestingly, the mechanism of the specific G·T repair strongly resembles that of G·U correction, in which the mispaired uracil residue is removed by a uracil DNA glycosylase⁶. The involvement of a glycosylase in the mammalian G·T repair seemed improbable, as the enzyme would have to differentiate between mispaired and paired thymines. But sodium hydroxide treatment of the nicked G·T duplex, incubated with the extract for 9 hours, gave rise to a small amount of a new oligonucleotide species which runs on a polyacrylamide gel between the *AccI* and *SalI* marker bands (Fig. 2c, lane 4). As this band was absent from the control lanes (Fig. 2c; lanes 2, 9 and 10), we take this result as evidence that it arose from the alkali-induced cleavage of the T strand containing a single apyrimidinic (AP) site at the position of the mismatch, to generate a labelled fragment with a 3'-terminal phosphate¹¹.

To further test our hypothesis that a glycosylase is involved in the removal of the mispaired thymine, we carried out the

nicking reaction under conditions that would inhibit most endonucleases and exonucleases, but not glycosylases, in the presence of 20 mM EDTA. Indeed, as Fig. 2c shows, the band that comigrated with the *AccI* marker became substantially less prominent under these conditions (lane 6). Instead, two other bands appeared, of which the upper could be converted to the lower by alkali treatment (lane 7). As heating with NaOH leads to the quantitative elimination of baseless sugars from DNA¹¹, the intensity of the lowest band in lane 7 reflects the total number of AP sites in the 5'-labelled T-strand. The fact that its intensity is approximately equal to that of the band produced after the same time period under the standard nicking conditions (Fig. 2c, lane 7; compare with lane 4) indicates that both species arose solely from the processing, chemical or enzymatic, of an AP site; that is, a glycosylase mediated the initial step in the specific G·T correction by removing the mispaired thymine.

To test whether the polymerase and ligase activities present in the nuclear extracts could repair the single nucleotide gap generated by the removal of the TMP, the gap-containing G·T duplex was subjected to a second incubation with the nuclear extract, this time in the presence of added ATP, Mg²⁺ and the four dNTPs. As shown in Fig. 3, the nicks rapidly disappeared during this treatment (lanes 2-5). In addition, when the treated duplex (shown in lane 5) was incubated with *SalI*, a proportion of it was cleaved (Fig. 3, lane 7). As the amount of the cleaved duplex was approximately equal to that of the nicked substrate before the second incubation with the extract (lane 2), we conclude that the single nucleotide gap in the duplex had been repaired.

What parameters determine the specificity and directionality of the G·T correction, in particular that of a thymine-specific glycosylase? It is conceivable that the recently identified HeLa protein of relative molecular mass 200,000 that binds exclusively to DNA containing G·T mismatches¹², could target it specifically to the mispaired ones. As such a mechanism has not yet been shown to act in mismatch correction¹³, this hypothesis remains to be experimentally substantiated. Nevertheless, it will be of interest to determine whether the repair of G·T and some G·A mispairs in *Escherichia coli*, mediated by the VSP¹⁴⁻¹⁶ and MutY^{17,18} pathways respectively, proceeds by a similar mechanism.

Like the VSP repair of *E. coli*, the mammalian G·T-specific repair pathway has probably developed to protect the cells from the deleterious effects of the spontaneous hydrolytic deamination of 5-methylcytosine. Our findings should enable us to characterize the enzymes involved in this process, which may play an important part in the maintenance of the differentiated phenotype of mammalian cells. □

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Avoiding false positives with PCR

S. Kwok and R. Higuchi

The exquisite sensitivity of the polymerase chain reaction means DNA contamination can ruin an entire experiment. Tidiness and adherence to a strict set of protocols can avoid disaster.

THE polymerase chain reaction (PCR)¹⁻³ is a powerful, exquisitely sensitive^{3,4} technique with applications in many fields such as molecular biology, medical diagnostics, population genetics and forensic analysis. The use of specific DNA amplification, as reflected by the number of publications reporting the use of PCR, has indeed grown "exponentially" in recent months. We and others⁵ are concerned, however, that some investigators may not be using adequate care in experimental design and execution when using PCR to detect only a few molecules of a target DNA sequence. A false positive or mistyping may occur when the majority of molecules to be detected arise from exogenous sources rather than from the sample itself.

Obviously, the fewer molecules one is trying to detect, the more one should guard against this possibility. The use of PCR for sensitive detection is complicated by the fact that the product of the amplification serves as the substrate for the generation of more product. A single PCR cycle produces very large numbers of amplifiable molecules that can potentially contaminate subsequent amplifications of the same target sequence. This kind of contamination has been termed PCR product "carryover" to differentiate it from contamination by naturally arising DNA (Fig. 1).

An analogy is useful to illustrate the scale of the contamination problem when using PCR to detect very few molecules. A typical PCR reaction can generate 10^{12} molecules of amplified DNA in a 0.1 ml reaction³. Imagine the uniform dilution of this number of molecules in a volume of

liquid that fills an Olympic-sized swimming pool (~50 m × 25 m × 2 m). A 0.1 ml aliquot of liquid from this pool would contain 400 amplifiable molecules.

To control contamination, one must prevent the physical transfer of DNA between amplified samples, and between positive and negative experimental controls. The following precautions have dramatically reduced false positive rates in our laboratories that perform PCR detection of HIV sequences⁶ (in which as few as 15 copies of DNA per sample are routinely detected). Those who use PCR for less demanding procedures, such as the preparation of DNA fragments from plasmid DNA templates, may be able to adapt a less stringent form of these guidelines with successful results.

Tips for better PCR

Physically isolate PCR preparations and products. To prevent carryover of amplified DNA sequences, we prepare samples in a separate room or biosafety hood from that in which the reactions are performed. The UV germicidal lamps in most biosafety hoods quickly damage any DNA left on exposed surfaces, making it unsuitable for subsequent amplification (G. Sensabaugh, personal communication), and further eliminating the possibility of contamination between samples. Separate sets of supplies and pipetting devices are dedicated for sample preparation and for setting up reactions.

Autoclave solutions. We routinely autoclave deionized water and those buffer solutions used in PCR and sample preparation that can be autoclaved without affecting their performance. Autoclaving under conditions that provide bacterial decontamination degrades DNA to a very low molecular weight (N. Arnheim, personal communication). We also autoclave disposable pipette tips and microcentrifuge tubes. Primers, dNTPs and *Taq* DNA polymerase cannot be autoclaved.

Aliquot reagents. We divide reagents into aliquots to minimize the number of repeated samplings necessary. All reagents used in the PCR are prepared, divided and stored in an area that is free of PCR-amplified product. Similarly, oligonucleotides used for amplification are synthesized and purified in a PCR product-free environment. It is advisable to record the lot(s) of reagents used so that if contamination occurs, it can more easily be traced.

Use disposable gloves. Our researchers

wear gloves and change them frequently, at the least when entering or reentering the isolation area. Changing gloves reduces the possibility of the transfer of amplifiable DNA from outside the isolation area and between samples.

Avoid splashes. Some types of sample tube have caps that require so much force to remove that liquid at the bottom of the tube may be splashed out. We use caps that do not require that much effort, and change gloves if such a splash occurs. It is also a good idea to spin any liquid down from the sides and top of the closed tube before attempting to open it.

Use positive displacement pipettes. The barrel of pipetting devices may become contaminated with aerosols containing sample DNA, leading to cross-contamination of samples. To prevent this, we use positive displacement pipettes with disposable tips and plungers.

"Premix" reagents. When possible, we mix reagents before dividing them into aliquots. All PCR reagents can be combined into a "premixure" which can then be pipetted into reaction vessels containing DNA. This minimizes the number of sample transfers and the chances for sporadic contamination. When dispensing the mixture, we pipette a "no DNA" negative control last so that it reflects the total reagent handled.

Add DNA last. Non-sample components, such as mineral oil, pre-mixed dNTPs, primers, buffer and enzyme, should be added to the amplification vessels before sample DNA. This minimizes cross-contamination by reducing the number of opportunities for the inadvertent transfer of DNA. After the addition of DNA, we cap each tube before proceeding to the next sample.

Choose positive and negative controls carefully. We do not use a highly concentrated solution of plasmid DNA containing the target sequence as our positive control, because it would introduce as many amplifiable molecules into the sample preparation area as a typical PCR. If plasmid DNA containing the target sequence is used as a positive control, it should be diluted substantially. Depending upon the detection system, as few as 100 copies of target may suffice as a positive control.

We include "no DNA" reagent controls and negative sample controls with each set of amplifications. The reagent controls should contain all the necessary components for PCR, except template DNA.

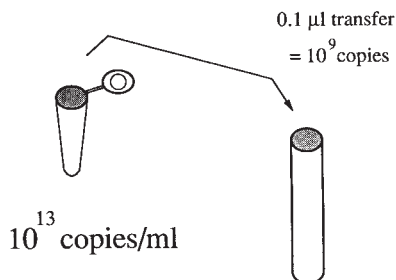


FIG. 1 The high concentration of amplifiable DNA in a completed PCR (typically ~ 10^{13} per ml) makes the inadvertent transfer of even a small volume to an unamplified sample quite serious. Reusing a pipette tip transfers 0.1 µl — roughly 10^9 copies of sequence. A microgram of human DNA contains only 1.4×10^5 copies of a single-copy gene.

Negative sample controls should not contain target sequences, but should have gone through all the sample preparation steps. Choosing negative controls for our HIV studies was complicated by the fact that PCR enabled HIV sequences to be detected from samples which were negative by all other tests. In the end, we used samples from low-risk individuals with well-known histories.

Avoiding other pitfalls

The amount of PCR product generated is sometimes insufficient, requiring reamplification after enrichment by gel electrophoresis. If the amount of amplified target sequence DNA in a particular gel slice is very low, one should be wary of cross-contamination from analogous PCR products or plasmid DNA containing the target sequence run in other lanes of the gel. To prevent carryover from equipment used in previous experiments, gel apparatus and combs should be soaked in 1 M HCl to depurinate any residual DNA, new razor blades should be used to excise each gel band, and the surface of the UV transilluminator should be covered with a fresh sheet of plastic wrap for each gel. Other potential sources of contamination include purified restriction fragments of target sequence, dot-blot apparatus, microtome blades, centrifuges, centrifugal vacuum devices and dry ice or ethanol baths. Most of these items are also amenable to treatment, if necessary, with 1 M HCl.

A final caution

If there is any doubt at all about a critical result, it is best to repeat the experiment again. The negative controls described above can rule out reagent contamination, but cannot guarantee against sporadic contamination events. Fortunately, the odds of a sporadic contamination event occurring twice the same way are very low. The net error rate of a series of tests is also usually lower than that of a single trial, even if the sporadic error rate of the repeated test is higher. PCR is simple and rapid, and consumes so little of most samples that repeat experiments can be performed, even in forensic work⁷.

Kits for performing PCR are available from Perkin-Elmer Cetus under the trademark GeneAmp. □

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At next week's BioExpo in Paris, France, new products range from an automated loop inoculation device to a crystallized Tris buffer preparation.

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Boehringer Mannheim is now offering its **Ultrapure Tris buffer in crystalline form** (*Reader Service No. 103*). The company says the buffer dissolves easier in solution than powdered buffers without clumping, and that it is free of contaminating proteases, DNases and RNases. At a purity of over 99.9 per cent, BMB says the Tris buffer is totally clear in solution, low in metal content and costs \$160 (US) for 5 kg.

A new range of supports for **ion exchange chromatography** is being added to the product list at IBF Biotechnics (*Reader Service No. 104*). The Trisacryl Plus supports, based on the Trisacryl matrix, are of fundamental benefit, says IBF Biotechnics: they are resistant to

extremes in pH, high flow rates, freezing, autoclaving and bacterial contamination. The Trisacryl Plus range is available in 300-ml bottles for the M grade (40-80 µm bead size) for \$118 (US) and in 1-litre bottles for the LS grade (80-160 µm) for \$355 (US).

Beckman Instruments has a new series of **HPLC autosamplers** that operate online as part of the company's System Gold series of liquid chromatographs or stand alone as part of another system (*Reader Service No. 105*). The model 507 autosampler is designed for laboratories that analyse large sample volumes on a daily basis. Operating features include a four-quadrant, refrigerated sample holder with a 96-vial capacity and a separate vial for rinse liquid. Vial access is direct, and column temperature control is available. The \$10,500-16,225 (US) model 507 also offers variable injection volumes, column switching, and pre-column reagent addition, mixing and reaction. □

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